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CHARACTERIZATION OF RECEPTORS FOR PERTUSSIS TOXIN

BY

© CLIFFORD G. CLARK

A THESIS SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

DEPARTMENT OF MEDICAL MICROBIOLOGY AND INFECTIOUS DISEASES

EDMONTON, ALBERTA

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Dr. Mark Peppler

Dr. Dennis Kunimoto

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This thesis is dedicated to my wife Rita, my children Carl, Eric, and Crystal, and to my parents, Glen and Cecelia

It is also dedicated to the memory of my grandparents, Clifford and Phyllis Lindell, whose profound love of nature and understanding of living things first kindled my interest in science

ABSTRACT

To elucidate the mechanisms by which pertussis toxin produces biological effects in different cell types, I have investigated eukaryotic cell receptors for the toxin. Affinity chromatography of solubilized membrane proteins from goose erythrocytes and T lymphocytes demonstrated that PT was capable of interacting with several proteins from each type of cell. Further investigations employed a photoaffinity labeling reagent, sulfosuccinimidyl 2-(*p*-azido-salicylamido)ethyl-1,3′-dithiopropionate, to identify a 70,000 Dalton pertussis toxin receptor on peripheral blood monocytic cells, T lymphocytes, and Jurkat cells. The use of a second photoaffinity labeling reagent, N-[4-(*p*-azido-salicyl-amido)butyl]-3′-(2′-pyridyldithio)propionamide, resulted in the identification of 50,000 and 32,000 molecular weight pertussis toxin receptors in addition to the 70,000 molecular weight protein.

Two sialic acid-specific lectins, the *Sambucus nigra agglutinin* and the *Maackia amurensis* leukoagglutinin, also photoaffinity labeled these receptors, suggesting they may be glycoproteins. Labeling of the three receptor proteins by the pertussis toxin-photoaffinity probe was reduced in the presence of these lectins or glycoproteins containing sialic acid while, in the reciprocal experiments, pertussis toxin abolished the labeling of these receptors by the lectins. These experiments supported the proposal that pertussis toxin was binding to the 70,000, 50,000 and 32,000 Dalton receptors in a lectin-like manner.

A photoaffinity labeling probe prepared using *Salmonella minnesota* wild type LPS labeled a Jurkat cell protein that appeared to be identical to the PT receptor on the basis of size and isoelectric point. LPS did not interfere with photoaffinity labeling of the 70,000 Dalton Jurkat cell receptor by PT, however, suggesting that this receptor either

had separate binding sites for PT and LPS or that the receptors for PT and LPS were different proteins.

Because the 70,000 Dalton pertussis toxin receptor had the same apparent molecular weight as serum albumin, I investigated the interaction of serum albumin with both pertussis toxin and the photoaffinity crosslinking agent. Serum albumin specifically bound the crosslinking agent but not PT. Antisera to serum albumins did not recognize the 70,000 Dalton PT receptor in immunoblots. Protein microsequencing of the receptor indicated it had a blocked amino terminus, unlike serum albumin. Therefore, it is unlikely that the 70,000 Dalton PT receptor is serum albumin.

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ABBREVIATIONS

ACT	adenylate cyclase toxin
APDP	N-[4-(*p*-azidosalicylamido)butyl]-3′-2(*p*-pyridyldithio)propionamide
ASD	2-(p-azidosalicylamido)1,3′-dithiopropionate
BSA	bovine serum albumin
CHO cells	Chinese hamster ovary cells
Con A	Concanavalin A
cpm	counts per minute
CR3	complement receptor 3
CT	cholera toxin
DNT	dermonecrotic toxin
DTH	delayed type hypersensitivity
EAMS	endotoxin activated mouse serum
ELISA	enzyme-linked immunosorbent assay
FBS	fetal bovine serum
FHA	filamentous hemagglutinin
FITC	fluorescein isothiocyanate
fMLP	formyl-met-leu-phe chemotactic peptide
Gal	galactose
GlcNAc	N-acetyl glucosamine
GPI	glycophospholipid inositol
G proteins	GTP binding proteins
HA	hemagglutination
HSA	human serum albumin
IC_{50}	concentration required for 50% inhibition of binding

Ig	immunoglobulin
IODO-GEN	1,3,4,6-tetrachloro-3α,6α-diphenylglycouril
kDa	kiloDaltons
LEC 2 cells	CHO cell variant lacking the terminal sialic acid on *N*-linked oligosaccharides
LEC 8 cells	CHO cell variant lacking the terminal sialic acid and galactose residues on *N*-linked oligosaccharides
LOS	lipooligosaccharide
LPS	lipopolysaccharide
MAL	*Maackia amurensis* leukoagglutinin
Man	mannose
MBq	megabequerel
NAD	nicotinamide adenine dinucleotide
NeuAc	sialic acid; neuraminic acid
NHS	*N*-hydroxysuccinimide
N-linked	asparagine linked
PBMC	peripheral blood monocytic cells
PBS	sodium phosphate buffered physiological saline
PBST	PBS containing 0.05% Tween 20
PE	phosphatidylethanolamine
PHA	phytohemagglutinin
PMA	phorbol myristic acid
PT	pertussis toxin
SASD	sulfosuccinimidyl 2-(*p*-azidosalicylamido)-ethyl-1,3´-dithiopropionate
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis

SNA	*Sambucus nigra* agglutinin
TCA	trichloroacetic acid
TCT	tracheal cytotoxin
TEMED	N,N,N´,N´-tetramethylethylenediamine
TLC	thin layer chromatography
T_s	suppressor T lymphocytes
two-dimensional SDS-PAGE	isoelectric focussing followed by SDS-PAGE
UV	ultraviolet
WGA	wheat germ agglutinin

CHAPTER I

BACKGROUND TO THESIS

A. The role of pertussis toxin in disease produced by *Bordetella pertussis*.

Whooping cough is a severe respiratory disease caused by the organism *Bordetella pertussis* (263). This disease continues to be a major cause of morbidity and mortality, accounting for 20 to 60 million cases among children each year worldwide with a 1% case fatality rate (27,274). Though the disease is most prevalent in developing countries, it continues to be a significant problem in developed countries as well. Vaccination with killed whole cell *B. pertussis* preparations has markedly reduced both morbidity and mortality in developed countries (25,27,263). These vaccines, however, produce a variety of side effects such as fever, muscle soreness, and local skin redness, and may additionally cause complications such as convulsions, hyporesponsive episodes, and encephalopathy (203). This fact has raised questions regarding the safety of the vaccine and led to lawsuits against companies producing it. Consequently, a great deal of research has been directed toward understanding the mechanisms of pathogenesis of *Bordetella pertussis* and the biological activities of macromolecules produced by the organism. The ultimate goals of this research are the development of better tools for the diagnosis of pertussis, the development of safer, more efficacious vaccines, and the production of new pharmaceutical agents for treating the disease in patients who, for one reason or another, are not protected by vaccination or who cannot complete the full course of immunization.

Bordetella pertussis produces a large number of secreted and surface-associated products that act as virulence factors. These include filamentous hemagglutinin (FHA), pertussis toxin (PT), adenylate cyclase toxin (ACT),

pertactin (69 kDa protein), dermonecrotic toxin (DNT), tracheal cytotoxin (TCT), fimbriae (agglutinogens), and lipooligosaccharide (LOS) (268). Of these, PT and FHA appear to be the most important for mediating *Bordetella pertussis* colonization of the respiratory epithelium and adherence to macrophages in humans (197,207,208,254,255). Many of the clinical manifestations associated with the disease occur at sites distant from the site of colonization. Since most of these effects are caused by PT alone, Pittman has proposed the concept of pertussis as a toxin-mediated disease (186,187). Furthermore, expression of the toxin correlates with pathogenesis in genetically modified organisms (143,268,269), and PT allows the bacteria to evade host defenses through its actions on the host immune system (268). Both PT and FHA have been combined to produce effective acellular vaccines against pertussis (27,107,113,130, 192,193), though the efficacy of these vaccines is still under scrutiny (46,74,170). While most vaccine development has focused on producing inactive PT with a genetically modified A (enzymatic) subunit, some investigators (7,219) have proposed developing a vaccine based on reactivity to the pentameric B oligomer of PT, which is involved in binding to eukaryotic receptors. As a result, a great deal of work is currently being done to elucidate structure-function relationships of the PT B oligomer and the binding of this moiety to eukaryotic receptors.

PT has also been used extensively for the analysis of receptor function and signal transduction in eukaryotic cells (see Tables 1 and 2; also 34,49,66,117,125,145,168,196). In most cases, the toxin is effective at low concentrations against intact cells, presumably due to the ADP-ribosylation of GTP-binding (G) proteins by the enzymatic subunit of the toxin. Though PT must bind to eukaryotic cells with relatively high efficiency in order to introduce the enzymatic subunit into the cytoplasm, almost nothing is known about the plasma membrane receptors responsible for PT

binding. Additionally, binding of the toxin may be sufficient to produce intracellular signals under some circumstances with some types of cells. To fully understand PT's effects in these various systems, therefore, we must determine the molecular mechanisms of toxin binding and translocation into cells.

B. Structure of PT.

PT is an A-B type toxin that can be dissociated into an enzymatically active (A) subunit, termed S1, and a pentameric binding (B) oligomer, which contains subunits S2, S3, S4, and S5 (240,257). Exposure of the holotoxin to 5 M urea causes the dissociation of S1 and S5 from the complex, leaving two dimers. These are dimer 1, which contains subunits S2 and S4, and dimer 2, which contains subunits S3 and S4; the stoichiometry of subunits in the intact B oligomer is therefore 1:1:2:1 for S2, S3, S4, and S5, respectively. In the holotoxin, the dimers are thought to be connected via the S5 subunit, although this remains to be verified by X-ray structure determination.

Electron microscopy of isolated PT reveals that the holotoxin takes the form of round particles or ring shaped doughnuts approximately 75 to 80 Å in diameter, each containing four to five subunits approximately 28 Å in diameter (152). A central hole is usually seen in the doughnut structures, and projecting spikes are also occasionally observed. Unfortunately, although PT has been crystallized (5), a crystal structure of the toxin has not yet been published. Irons and colleagues, using spectroscopic and conformational studies of PT, have determined that the toxin is composed mostly of β-sheet and β-turn structures, with very little α-helix (212). Interestingly, the toxin has a high proportion of solvent-exposed tyrosines.

The operon coding for the PT subunit genes has been sequenced, the protein sequences have been determined, and

the requirements for expression of the toxin have been elucidated (65,129,173,174,191). The PT gene has been expressed in *Bacillus* and *E. coli* (18,204), facilitating studies designed to determine the requirements for secretion of the toxin (190) and allowing elucidation of amino acids involved in the ADP-ribosylation and NAD-glycohydrolase activities of the toxin (17,31,40,41,123,128,189). Comparison of S2 and S3 sequences with eukaryotic binding proteins is also helping to define the PT motifs involved in binding to glycoproteins and receptors (33,194,207).

The PT S1 subunit catalyzes the transfer of the ADP-ribose moiety from NAD to a cysteine residue in the carboxy terminus of $G\alpha i$, $G\alpha o$, and $G\alpha p$ proteins, and is also capable of hydrolyzing NAD in the absence of the ADP-ribose acceptor (29,85,101,104,156,240)). In addition, PT can induce changes in the α subunits of G proteins independently of ADP-ribosylation (199). The ADP-ribosylation of G proteins, and possibly also the NAD-independent modification of G proteins, results in the uncoupling of these proteins from cell signalling pathways, interfering with receptor stimulated signalling activity (see Gill (59) and Tables 1 and 2). In order for the S1 subunit to reach its intracellular target, the B oligomer of PT must first bind to specific cell-surface receptors and facilitate the translocation of S1 through the membrane (175-177,241).

C. PT binding to glycoproteins and eukaryotic cells.

1. Specificity of the interaction of PT with glycoproteins.

Many affinity chromatography methods used to purify PT exploit the ability of the toxin to bind to human haptoglobin (33,90,206) or fetuin (39). PT interacts with the *N*-linked oligosaccharide structures on fetuin in a lectin-like manner (8,213). Apparently, fetuin's *O*-linked oligosaccharides are not involved with binding. Sekura and Zhang (213) suggested that the *N*-acetylglucosamine-mannose (GlcNAc-Man) bridge

structures in these oligosaccharides are important for PT binding. More recent data (8,75) demonstrate that the α(2-6)-linked sialic acid and galactose residues are also important for the optimal binding of PT. Exoglycosidase enzymes were used to sequentially remove sugars from the *N*-linked oligosaccharides of fetuin. Though the removal of sialic acid residues from fetuin decreased the binding activity of PT in polystyrene binding assays, removal of the galactose residues partially restored binding (8). Addition of galactose and sialic acid to these incomplete oligosaccharide structures using glycosyltransferase enzymes restored the full PT binding of fetuin, though sialic acid was added only in the α(2-6)-linkage (8). Furthermore, high concentrations of sialic acid (NeuAc) and sialyllactose partially inhibited PT binding to fetuin (273). All these observations are consistent with the hypothesis that PT may have an extended binding site recognizing more than one sugar in the oligosaccharide chain. The observation that the proteolytic degradation of fetuin greatly reduces its ability to bind to PT suggests that the target oligosaccharide must be in the correct stoichiometry or spatial orientation for binding (8).

PT is capable of binding with varying affinities to a number of glycoproteins containing *N*-linked oligosaccharides, including (roughly in decreasing order of affinity) fibrinogen, laminin, fetuin, haptoglobin, ceruloplasmin, α-acid glycoprotein, thyroglobulin, and transferrin (75,273). These studies have shown that PT is capable of binding to glycoproteins that contain only biantennary structures as well as those that have tri- and tetra-antennary structures. There is, however, a striking (140 fold) difference in the affinity of PT for fibrinogen and transferrin, even though both glycoproteins have identical biantennary *N*-linked oligosaccharides (75). This difference in affinity may arise partly from the fact that fibrinogen has four biantennary structures per molecule while transferrin has only two, and

partly from differences in the spatial orientation of these oligosaccharide structures on the two glycoproteins.

Isolated dimer 1 and dimer 2 of PT exhibit differences in glycoprotein binding. In dot blot assays, dimer 1 binds better to fetuin, haptoglobin and thyroglobulin than dimer 2, while dimer 2 binds better to laminin and asialofetuin (28,273). Dimer 1, but not dimer 2, is retained on haptoglobin columns in the presence of 5 M urea during affinity chromatography of dissociated PT (33). Schmidt and Schmidt (210) produced antibodies to the S2 subunit that do not cross-react with S3. Though the hemagglutinating and fetuin-binding activities of PT are totally abolished by these anti-S2 antibodies, they have no effect on CHO cell clustering. These observations indicate that dimers 1 and 2 interact differently with eukaryotic cells as well as with glycoproteins.

2. Hemagglutination.

PT is capable of hemagglutinating erythrocytes from a number of species, including ox, goose, horse, monkey, sheep, chicken, and rabbit (67,206). Goose and horse erythrocytes are agglutinated by relatively low concentrations of PT (0.5 µg/ml; 90), while ten times higher concentrations of PT are required for the agglutination of chicken erythrocytes. Chymotrypsin-treated goose erythrocytes are 30 times more sensitive than untreated erythrocytes (9). Hemagglutination provides a relatively fast and simple means for detecting PT in biological samples (9,89).

The hemagglutinating activity of PT resides in the toxin's B oligomer (177). Dimer 1 is as effective as the holotoxin or the B oligomer, while dimer 2 is somewhat less effective. Hemagglutination presumably requires at least two binding sites on each agglutinating molecule, though it is not clear if all the binding sites present in the isolated dimers are exposed in the B oligomer or the holotoxin. Surprisingly, the hemagglutination of erythrocytes by PT is

not attenuated by acetamidination, which abolishes many of the other activities of the toxin not directly related to its enzymatic activity (177). This suggests that the binding site(s) on PT for erythrocytes may have only partial identity with the binding site for other eukaryotic receptors.

The PT-mediated hemagglutination of goose erythrocytes is not inhibited by 100 mM concentrations of most sugars and glycosides, including lactose and *N*-acetyllactosamine (8). In contrast, chitobiose and sialyllactose at a minimum concentration of 50 mM inhibited this activity, supporting the hypothesis that PT binding to erythrocytes and fetuin shows similar saccharide specificity. A series of experiments by Schmidt and colleagues confirms and extends these observations. These investigators have generated a series of peptides to the S2 and S3 subunits of PT and have made antibodies to these peptides (209-211). Antibodies that recognize three linear S2 peptides, comprising amino acids 1-7, 35-50, and 91-106, block PT binding to fetuin and the hemagglutination of goose erythrocytes (210). Similarly, antipeptide antibodies directed at the S3 linear sequences containing amino acids 1-12, 12-23, 14-29, and 36-51, inhibit the hemagglutination of goose erythrocytes (209). It is intriguing that the peptides from both S2 and S3 that contain amino acids 35-52 were found to be important for binding to erythrocytes and fetuin by these investigators and to ciliated epithelial cells and macrophages by Tuomanen and colleagues (207). In contrast to the results of acetamidination experiments discussed in section I.C.3., these data suggest that PT may use the same binding site to adhere to glycoproteins, erythrocytes, and eukaryotic cells. One possible explanation for the difference is that the binding sites on PT responsible for hemagglutination have only partial identity with the binding sites responsible for binding glycoproteins and eukaryotic cell-surface receptors. This theory is supported by the data, presented above, that indicates some linear peptides important for hemagglutination

are different in subunits S2 and S3, though it is not known whether these peptides combine to form a conformational erythrocyte-binding epitope. Alternately, acetamidination may cause gross structural changes in PT that affect eukaryotic cell and glycoprotein binding more that hemagglutination.

3. PT binding to other eukaryotic cells.

PT acts as an adhesin that mediates attachment of *Bordetella pertussis* bacteria to respiratory epithelium and macrophages (197,208,254). The ability of lectins and carbohydrates to inhibit the adherence to ciliated epithelial cells, suggests that the PT receptor(s) on these cells contain saccharide structures (255). Furthermore, intact bacteria are capable of binding to glycolipids separated on thin layer chromatography (TLC) plates (207,255). Though Brennan *et al.* could not detect binding of PT to gangliosides or neutral glycolipids on TLC plates (24), Tuomanen and colleagues found that the isolated toxin subunits S2 and S3 have different binding specificities for different glycolipids, with S2 binding to (ciliary) lactosylceramide and S3 binding to (leukocytic) gangliosides (207). In addition, they found that a Gly-Asp-Thr-Trp finger sequence (present in the sequence containing amino acids 37 to 52), present in slightly different forms in both subunits, is responsible for this binding specificity. An antibody that was capable of blocking S2-mediated adhesion of *B. pertussis* bacteria to cilia recognized glycolipids in the cilia as well as a single ciliary glycoprotein (255). These data suggest that PT may bind to both glycolipids and glycoproteins that have similar oligosaccharide structures. Karlsson (98) has proposed that many of the interactions of adhesins with glycolipids may be of low affinity, though of somewhat higher avidity, and that these interactions may contribute significantly to the adherence of bacteria to cells as a second step after an initial, high affinity binding process. The finding that rather high concentrations of lactose and

galactose are necessary for the 50% inhibition of PT-mediated *B. pertussis* binding to ciliated respiratory epithelial cells (255) is consistent with this hypothesis. In this context, the binding of PT to glycolipids may facilitate the interaction of the toxin with the eukaryotic membrane and aid toxin entry into cells.

Low concentrations of PT affect Chinese hamster ovary (CHO) cells, causing them to clump and enhancing the cAMP response after stimulation by cholera toxin or prostaglandin E_1 (78,79). Basal cAMP levels are unaffected by PT treatment of these cells. The cell clustering effect, at least, is due to the ADP-ribosylation of G proteins in the CHO cells (29). PT and its isolated B oligomer bind to a 165 kDa glycoprotein as well as a protein of about 70 to 85 kDa in CHO cell extracts separated by gel electrophoresis and transferred to nitrocellulose filters (24). Sialidase treatment of the 165 kDa glycoprotein results in the loss of PT binding, indicating that PT is binding to this protein through lectin-like interactions similar to those discussed above. CHO cell mutants lacking the terminal sialic acid (LEC 2 cells) or both the terminal sialic acid and galactose residues (LEC 8 cells) exhibited reduced binding to, and intoxication of, the cells by PT (273). Higher concentrations of PT were required for intoxication of the LEC 2 cells (250 ng/ml) than the LEC 8 cells (31 ng/ml), while wild type CHO cells were clustered by a minimum concentration of 0.06 ng/ml of the toxin (273). This pattern of binding is similar to the pattern of binding of PT to intact fetuin, fetuin lacking sialic acid, and fetuin lacking sialic acid and galactose (8), further supporting the hypothesis that PT binds to eukaryotic receptors and glycoproteins through similar binding sites.

PT intoxication of CHO cells is not inhibited by an antibody capable of inhibiting the adhesion of *B. pertussis* to cilia (255) or by antibodies to S2 peptides that are capable of inhibiting PT binding to fetuin and goose erythrocytes (210). However, a murine monoclonal antibody

that recognizes a peptide corresponding to amino acids 107-120 in PT's S2 subunit inhibits PT binding and CHO cell clustering (264). The situation is further complicated by the observation that antibodies which recognize conformational epitopes on PT's S3 subunit inhibit the binding of dimer 2 (S3-S4) and the PT holotoxin to CHO cells, but do not inhibit the binding of dimer 1 (S2-S4) (120). It is possible that the PT holotoxin contains several binding sites and that a PT binding epitope distinct from the one recognized by fetuin and goose erythrocytes may be involved in the interaction of PT with CHO cells. Is is also possible that the dissociation of PT into dimers exposes binding sites on each dimer that are not available in the holotoxin.

The leukocytosis produced in mice by PT is prevented by immunization of the animals with the toxin's B oligomer or with isolated dimers, indicating that some component of the B oligomer is also involved with toxin binding to lymphocytes (6,71). Monoclonal antibodies directed to the S3 subunit strongly inhibit the leukocytosis-promoting, histamine-sensitizing, and CHO cell-clustering effects of PT, indicating that dimer 2 (S3-S4) is important for all three activities (68,108).

Acetamidination of lysine residues in PT differentially affects many of the *in vitro* and *in vivo* activities of the toxin (175,177). Similarly, reductive methylation of free amino groups in dimer 2 (S3-S4), but not dimer 1, impairs the ability of PT to cause lymphocytosis and mitogenesis of lymphocytes and inhibits the PT-mediated stimulation of glucose oxidation in adipocytes (176). In contrast, dimer 1 (S2-S4) is much more effective than dimer 2 in stimulating glycerol release from adipocytes, and methylation has no effect on this activity of dimer 1 (176). In fact, membrane adenylate cyclase activity, insulin secretion by islets, and glycerol release by adipocytes are all affected only slightly by methylation. These data suggest that PT may bind to the cell surface through different mechanisms, perhaps even to

different receptors, and that the particular receptor bound may determine the activity that will be affected.

4. Evidence for two step binding of PT to eukaryotic cells.

Early investigations into the lymphocytosis-promoting activity of PT suggested that the toxin can associate reversibly with erythrocytes and lymphocytes (91) in a time and temperature-dependent manner, and that this association can be reversed by the addition of fetal bovine serum (FBS) (1). Immediate transfer of PT-treated mouse erythrocytes or lymphocytes into mice stimulates the characteristic lymphocytosis, suggesting that the PT introduced on the injected cells can affect endogenous lymphocytes in the animal. Subsequent investigations using cells that had been treated with PT for longer periods of time (225,247) indicated that PT binding to lymphocytes was not reversible. These observations are consistent with a two-step model of PT binding, in which the toxin first associates with specific receptors in a reversible manner and subsequently binds irreversibly to the membrane. This latter step could involve the introduction of the enzymatic subunit into the membrane.

The mechanism responsible for the introduction of PT into membranes is not well characterized. Cytochalasin B and NH_4Cl have no effect on PT action, indicating that an acidic environment is not a requirement for PT entry and that endocytosis is not involved (32). Furthermore, hydrophobic photolabelling studies have shown that subunits S2 and S3 of the PT B oligomer can associate through hydrophobic interactions with detergent micelles (144), consistent with the presence of long hydrophobic stretches of amino acids in these subunit proteins (129,174). The toxin is not soluble in water, tending to form insoluble aggregates, while the B oligomer forms insoluble aggregates even in the presence of 2 M urea (240). Aggregation could also be partly the result of electrostatic interactions due to the high isoelectric points

of the toxin subunits comprising the B oligomer (240). However, the fact that PT can be isolated using hydrophobic chromatography and that 10% glycerol is necessary to prevent the irreversible adsorption of the toxin to plastic surfaces (238) suggest that the hydrophobicity of PT is an important property for both aggregation of the toxin and for interactions with host cell membranes. It is possible that binding of PT to receptor glycoproteins or glycolipids on host cells induces a conformational change that further enhances the hydrophobicity of the toxin and leads to irreversible insertion of the toxin into the membrane.

Detergents, phospholipids, and ATP bind to PT, inducing conformational changes responsible for the release of the S1 enzymatic subunit from the B oligomer (30,154) and enhancing the ADP-ribosylation of PT targets (126). The ATP binding site is located in the B oligomer of the toxin; binding is dependent on the conformation of the B oligomer within the holotoxin, since phosphate ions or the S1 subunit stabilize the toxin in a form that binds ATP with high affinity (95). Hausman et al. (95) have therefore proposed a model for PT action in which the B oligomer binds to the surface of eukaryotic cells and subsequently becomes exposed to the interior of the cell. ATP then binds to the toxin's B oligomer promoting the release and subsequent activity of the ADP-ribosyltransferase. Interestingly, antibodies specific for subunit S4 or for a conserved epitope on subunits S2 and S3 interfere with the enzymatic activity of PT (120), perhaps by preventing the dissociation and subsequent activation of the S1 subunit.

The irreversible binding of PT to cells as a result of the insertion of the toxin into cell membranes may cause problems in interpreting the inhibition of CHO cell clustering using glycoproteins. Even in the presence of reversible, competing interactions, this irreversible binding of PT should shift the equilibrium in favor of toxin binding to cells during the relatively long incubation times used for

performing the CHO cell assay. In the absence of data that take this effect into account, it is difficult to draw conclusions from the CHO cell assay regarding whether the binding site(s) in PT for glycoproteins and cells are different or the same. To address this question in the future, we have developed a biotinylated derivative of PT for the direct assessment of PT binding to cells (this work).

D. Effects of PT on T lymphocytes.

Though PT's effects on T lymphocytes have been extensively reviewed (66,92,156,157,159,186,187,265), our understanding of the molecular mechanisms responsible for these effects is increasing rapidly. In addition, the identification of cellular receptors for PT (this work) will allow entire signalling pathways to be elucidated. A re-evaluation of the extant information regarding PT's effects on these cells is therefore of value. For aspects of PT's effects on the immune system not covered here, the reader is referred to the comprehensive review by Munoz (157).

1. Lymphocytosis.

Bordetella pertussis infection of humans causes a striking lymphocytosis, first described in 1897 by Fröhlich [(51), cited by Morse, (149)]. This effect is also seen in several other species, including mice (149), calves, sheep (195), rats and guinea pigs (93) after intravenous injection of the whole cell pertussis vaccine. The lymphocytosis does not result from an increase in the rate of lymphocyte production (153), and it can be abrogated using specific antiserum (91). Leukocytes released from the bone marrow (153), thymus, and lymph nodes (151) accumulate in the blood (149), lungs (225,250-252), and spleen (226) of affected animals. Though most investigators attribute the lymphocytosis seen after *B. pertussis* infection to the accumulation of normal-appearing mature small lymphocytes, a recent report describes the appearance of lymphocytes with

abnormal nuclei and terminal deoxynucleotidyl transferase activity in response to infection with this organism (114).

The factor responsible for lymphocytosis and leukocytosis, PT, was purified by Morse and Morse (152), and was also shown to produce histamine sensitization and hypoglycemia in mice. Spangrude *et al.* (225) confirmed that PT is responsible for the lymphocytosis produced in mice (151). The mobilization of T lymphocytes and thymocytes from lymphoid organs provides the major contribution to the prolonged accumulation of leukocytes in the blood (114,225), though the release of B cells from these organs (12,152,157) and the inhibition of neutrophil migration to peripheral sites of inflammation are also involved (195,226). PT inhibits antigen-specific contact hypersensitivity after the adoptive transfer of PT-treated lymphocytes, though the transfer of these treated cells directly into the site of antigen exposure overcomes this inhibition (224). Lymphocyte extravasation into non-lymphoid tissue such as skin is therefore also PT sensitive.

Sugimoto *et al.* (236) developed an *in vitro* assay to assess the effects of PT treatment on lymphocyte migration through thymic epithelial cell layers. A concentration of 0.1 ng/ml of PT causes nearly maximal inhibition of lymphocyte traffic across the monolayer in the normal direction and increases the number of lymphocytes traversing the monolayer in the reverse direction. These results, reproduced by Verschueren et al. (259) using monolayers of fibroblast-like mouse embryo (10T1/2) cells, indicate that the homing process inhibited by PT is highly asymmetric. The invasion of hepatocyte cultures by malignant lymphoma and T cell hybridoma cultures is similarly inhibited by PT (201), though the *in vivo* PT inhibition of liver invasion and metastasis by these cells depends on the cell line used.

Murine lymphocytes pretreated *in vitro* with PT and transferred to untreated mice remain in the blood, while normal lymphocytes introduced into PT-treated mice localize

normally into lymph nodes or Peyer's patches (150,225). Furthermore, when mice are injected simultaneously with differentially radiolabeled PT-treated and untreated lymphocytes, the untreated cells localize in lymphoid organs whereas the PT-treated cells remain in the blood (215). Together, these results indicate that the effect of PT is on lymphocytes and not on the high endothelial venule (HEV) structures responsible for lymphocyte homing. Lymphocytes treated *in vitro* with PT still adhere to HEV in frozen lymph node sections (225), and this interaction is readily inhibited by antibodies specific for the lymphocyte receptor that recognizes endothelial cells (54). Finally, PT-treated T lymphocytes were shown to retain normal levels of the MEL-14 homing receptor responsible for HEV binding (86,229). The loss of extravasation potential of lymphocytes that leads to lymphocytosis is therefore not due to changes in the ability of these cells to bind to homing receptors.

The inability of PT-treated lymphocytes to extravasate into tissues correlates with the inhibition of lymphocyte chemotaxis to casein (229), IL 8, and fetal bovine serum (FBS) (10,15,271) in response to low doses of PT. Random motility and directed migration of these cells are both inhibited in a parallel, dose-dependent fashion (226). This inhibition also correlates with the dose-dependent inhibition of polarization responses of T lymphocytes (271). High-walled endothelial cells in culture produce a high molecular weight product responsible for inducing the polarization of lymphocytes that are in solution or bound to these endothelial cells. Preincubation of lymphocytes with as little as 4 ng/ml PT inhibits this shape change (69). The dose- and time-dependence of the PT inhibition, as well as the low doses needed, are consistent with a mechanism of toxin action dependent on the ADP-ribosylation of G proteins.

The effect of PT on lymphocyte homing does not appear to be associated with easily detectable changes in cell function or signalling. Since the adherence of lymphocytes to HEV

requires normal metabolic and microfilament activity, and since adherence is not affected by PT treatment, the effects of PT cannot be explained by gross changes in either metabolism or microfilament function (54). Basal cyclic AMP (cAMP) levels in PT-treated lymphocytes are not significantly different from those in untreated cells (37,226,258), though treatment of Jurkat cells with 200 ng/ml PT for 4 hours sometimes inhibits and sometimes enhances agonist- and cholera toxin-mediated cAMP accumulation (258). Similar PT treatments using concentrations as low as 5 ng/ml inhibit the prostaglandin E_2-mediated cAMP accumulation in Jurkat cells and human lymphocytes (185,258). Incubating PT-treated lymphocytes with an inhibitor of the activation of protein kinase C (H7) does not affect the loss of extravasation potential by these cells, suggesting that activation of protein kinase C is also not involved in this phenomenon (86). PT does cause a small decrease in the membrane fluidity of treated lymphocytes (229), but it is not yet clear whether this property of the toxin has any biological significance.

Several lines of evidence support the idea that the effect of PT on lymphocyte extravasation is the result of the ADP-ribosylation of a PT-sensitive G protein (for instance $G\alpha i$). Monoclonal antibodies specific for the S1 subunit of PT neutralized the leukocytosis-promoting activity of the toxin (205). The modification of amino groups in PT abolished both the binding and lymphocytosis-promoting activity of the toxin while leaving the *in vitro* enzymatic activity of the toxin intact (175), confirming that the B oligomer of the toxin is necessary for the introduction of the S1 subunit. Spangrude *et al.* (225) reported that the half-maximal inhibition of lymphocyte homing in mice was achieved using 4.5 ng/ml PT and a 2 hour preincubation of cells, or 0.2 ng/ml PT and an 18 h preincubation of cells. A minimum of 1 to 3 hours of incubation was required for this effect to become apparent, and treated lymphocytes injected into mice before this time localized normally into lymphoid organs. Similar

concentrations (0.1 ng/ml PT) are required for the maximal inhibition of chemoattractant-induced lymphocyte migration *in vitro* (15,236), though the dose required for 50% inhibition of lymphocyte polarization is slightly higher (18 ng/ml using IL 8 and 42 ng/ml using FBS as attractants) (271). Sewell and Andrews (215) have calculated that the dose of their PT preparation that caused lymphocytes to lose the ability to home corresponded to about 200 molecules of PT per cell, approximately two orders of magnitude lower than the amount of PT that caused mitogenesis of T cells in their system. The low concentrations of PT required for effectiveness and the time lag necessary for the effects of the toxin to become apparent are characteristic of G protein involvement.

The expression of the gene for the PT S1 subunit in transgenic thymocytes causes a profound depletion of $G_{\alpha i}$ proteins and a lack of emigration by these cells to secondary lymphoid organs (36,37). Indicators of lymphocyte function such as the presence of mature T cell surface markers and the increase of intracellular calcium levels and IL-2 production upon activation were normal in these experiments. PT also inhibits IL-1 induced [^{35}S]GTPγS binding and [γ-^{32}P]GTP hydrolysis in membranes of a murine thymoma line, suggestive of G protein involvement (179). Finally, genetically modified PT holotoxin variants lacking ADP-ribosyltransferase activity do not induce leukocytosis (21,107,130,192), further supporting the hypothesis that PT modification of cellular G proteins is responsible for the effect.

2. Mitogenesis.

T lymphocytes from rats, mice, and humans (111,148,260) proliferate and produce IL-2 in response to PT stimulation. Human T lymphocytes can be stimulated with lower amounts of PT and have a broader concentration optimum for stimulation than T lymphocytes from rats and mice (260). In addition, cells from different human donors respond to different

concentrations of PT (260). The mitogenic stimulation of T lymphocytes by PT can be separated from the specific stimulation of these cells obtained after immunization of mice with PT (48), indicating that the mitogenic effect is not the result of T cell activation through the T cell receptor (TCR)/CD3 complex by antigen bound to MHC molecules. PT does not specifically expand a subset of T cells bearing certain Vβ elements after *in vivo* or *in vitro* treatment of murine lymphocytes (96). It is therefore not a bacterial superantigen, which act by stimulating T lymphocyte proliferation through non-MHC-haplotype restricted binding to the MHC class II molecule. Furthermore, the isolated B oligomer of PT is sufficient to induce mitogenesis, though neither dimer 1 nor dimer 2 are mitogenic by themselves, suggesting that cross-linking of cell-surface receptors via the divalent binding of PT is required to produce the effect (169,241). Though cross-linking of many T lymphocyte cell-surface receptors, including CD2, CD3, CD4, CD5, CD6, CD7, CD8, Tp44, and CDw18, is known to result in cell proliferation (122), the receptors involved in the response to PT have not been unambiguously identified.

The role of the T lymphocyte-specific CD3 antigen in mitogenic stimulation by PT is currently under investigation. While Gray *et al.* (61) found that PT caused increased intracellular calcium concentrations only in $CD3^+$ cells, Macintyre et al. (134) observed calcium mobilization in a $CD3^-$ cell line. Kamradt *et al.* (96) have also questioned whether the TCR/CD3 complex is involved in PT mediated activation of T lymphocytes, since this stimulation was independent of the Vβ TCR present. It would therefore seem unlikely that PT interacts directly with CD3, though more work needs to be done to determine whether CD3 has a role in signalling.

Other cell-surface molecules have been implicated in the PT-mediated activation of T lymphocytes. Rogers *et al.* (200) identified a 43 kDa PT-receptor on Jurkat and HPB-ALL cells.

In the same study, however, these authors failed to demonstrate any PT receptor species on neutrophils, which are known to respond strongly to low doses of PT (19). I have been unable to demonstrate the presence of a 43 kDa T lymphocyte or Jurkat cell receptor despite using a very similar photoaffinity labeling procedure, though I have demonstrated that three proteins with molecular weights of approximately 70 kDa, 56 kDa, and 32 kDa are photoaffinity labeled using ^{125}I-ASD-PT (this work). Using glycoproteins and lectins with well-defined binding specifities, I have further shown that some component of the T lymphocyte receptor for PT is a sialylated glycoprotein. This information is consistent with the known substrate requirements for PT binding (see above) and with the fact that T lymphocytes require properly processed *N*-linked oligosaccharides on cell-surface receptors for full activation (249).

A dose of 250 ng to 500 ng of PT per 5 x 10^5 cells is sufficient to produce the mitogenic effect of the toxin (62,111,134,171,233,234) as long as accessory cells such as B lymphocytes (82) or monocytes (62,99,100) are present. This requirement for accessory cells is not shared by other T cell mitogens, such as phytohemagglutinin (PHA) or Concanavlin A (ConA) (110,265). Phorbol myristic acetate (PMA) or IL-2 can substitute for accessory cells to stimulate the growth of colonies of monocyte depleted T lymphocytes *in vitro* (100,234), suggesting that a specific combination of signalling events is responsible for mitogenesis and that PT provides only part of the required stimulus. The dose-response curve for PT-induced mitogenesis of T lymphocytes peaks at 2 to 4 µg/ml and returns to baseline at 8 to 10 µg/ml (148), as would be expected if mitogenesis resulted from crosslinking of T cell-surface receptors. At high PT concentrations, the toxin should exhibit monovalent binding to receptors and consequently be unable to produce the response.

PT treatment of human lymphocytes causes a rapid elevation in cytoplasmic calcium levels that results primarily from an influx of extracellular calcium through ion channels (233). This calcium influx is stimulated in a dose-dependent manner by equivalent concentrations of PT or the toxin's B oligomer (202). The rapid, repetitive spiking pattern of intracellular calcium changes produced by PT in single cell experiments is different than the sustained changes resulting from the activation of T cells with other mitogens or with anti-CD3 antibodies (231), suggesting different cellular signalling pathways may be involved. Gardner (56) has recently suggested that the frequency of calcium oscillations may encode important signalling information in T lymphocytes. The influx of calcium is preceded by the release of smaller amounts of calcium from intracellular stores and a concomitant rapid cytoplasmic alkalinization (62,134). Increases in inositol phosphate levels in response to PT treatment have been detected in Jurkat cells (231), but not in peripheral human T lymphocytes (234). Nonetheless, it remains possible that the initial release of calcium from intracellular stores is stimulated through an inositol phosphate-dependent signalling pathway (231).

Rosoff *et al.* (202) reported that treatment of Jurkat cells with mitogenic doses of PT caused increases in intracellular calcium, diacylglycerol, and inositol triphosphates, leading to the stimulation of IL-2 production. This is consistent with data indicating that PT activates protein kinase C in Jurkat cells (248). PT also activates a tyrosine protein kinase in Jurkat cells in a manner similar to the lectin, phytohemagglutinin (PHA). The PT concentrations required for half-maximal stimulation of calcium influx and tyrosine kinase activation in Jurkat cells are approximately 10^{-8} M, similar to the PT concentrations required for stimulation of mitogenesis (202,248). Surprisingly, mitogenic doses of PT inhibit changes in the

concentration of intracellular calcium in T lymphocytes stimulated by PHA or antibodies to the TCR/CD3 complex (61,121,172). Pretreatment of cells with PT for 1 hour or more is necessary for this effect to be seen. Treatment of cells with the B oligomer alone also induced a refractory state with respect to calcium influx (61,223), however, suggesting this phenomenon may be the result of a post-activation refractory state rather than the modification of specific G proteins (134). These data clearly indicate that PT binding to target cells can stimulate biological effects separate from those due to the enzymatic activity of the toxin.

The relevance of PT's mitogenic properties *in vivo* has been called into question by Podda *et al.* (193), who did not detect changes in the the total numbers and subpopulation proportions of T cells, B cells, or natural killer (NK) cells after injection of human volunteers with PT detoxified by genetically altering the S1 subunit. Furthermore, both the $CD4^+$ and $CD8^+$ human T cell subsets proliferate in response to PT (62), though one report suggests that PT may stimulate the expansion of the $CD4^+$ subset to a greater degree than the $CD8^+$ subset (100). It therefore remains possible that the mitogenic action of PT could affect the functioning of the immune system *in vivo*, even if mitogenic concentrations of the toxin were limited to specific areas of the body. Munoz and his colleagues have proposed that the lymphoid organs may provide a microenvironment for the induction of T cell proliferation by PT (217).

3. Other effects involving T lymphocytes: Adjuvant effects, delayed-type hypersensitivity, IgE production, and mitogen-induced IL-2 production.

PT can act as an adjuvant to increase the antibody response induced by specific antigens (124,156,160), and can also act to suppress antibody production (115,157). Differences in the timing of PT administration, mouse strain

used, and antigen used, all affect whether enhancement or suppression of antibody production will occur (157). When PT is administered before immunization with most antigens (for example, hen egg albumin), suppression of the IgG and IgM response to the antigen given is seen, while administration of PT at the time of or after immunization causes an increase in the titres of IgG, IgM, and IgE antibodies (157,160). Submitogenic doses of PT enhance antibody production, while mitogenic doses inhibit antibody formation. In addition, PT can stimulate antibody production through both a T cell dependent pathway, increasing titres of all antibody classes, and through a T cell independent pathway (direct stimulation of B cells), increasing titres of IgG_1 antibodies only (72). The enzymatic activity of PT is required for the expression of adjuvancy, since genetically engineered toxins with inactive S1 subunits are not able to mediate this effect (21). Doses as low as 4 ng of PT per mouse are fully effective in promoting the adjuvant effect (160), further supporting the hypothesis that it is the result of ADP-ribosylation of G proteins by the toxin. Murine thymocytes treated with mitogenic doses of PT mediate suppression of specific antibody responses (115), and similar doses of PT enhance gamma interferon (IFN-γ) production by lymph node and spleen cells (216,261). Together, these results suggest that PT may preferentially activate either the Th_1 or Th_2 (T helper) lymphocyte subsets. The timing of PT addition, combined with other (unknown) factors, could result in the differential activation of Th_2 cells, with the resulting production of cytokines that enhance antibody production by B cells, or in the differential activation of Th_1 cells, resulting in IFN-γ production and inhibition of help for antibody responses. The adjuvancy of PT may be also be due to the differential activity of the toxin on $CD8^+$ T suppressor (T_S) cells as well as $CD4^+$ T amplifier cells. Using the Th independent antigen, type III pneumococcal polysaccharide, Hasløv *et al.* demonstrated that PT causes the selective

inactivation of T suppressor cells and the induction of an increased frequency of T amplifier cells (70). Though the basis for these effects is not clear, the observation that PT and Con A differentially affect the antibody responses to this antigen suggests that they are not due to to the general mitogenic action of PT.

Further evidence for the differential activation or suppression of T lymphocyte subsets comes from the examination of the effects of PT on antigen-specific delayed-type hypersensitivity (DTH) in the mouse (157,181). The transfer of PT-treated Lyt-1^+ (mouse helper) T cells results in the transfer of DTH, while the transfer of Lyt-2^+ (mouse suppressor) cells does not (244). As with the adjuvant effect, the timing of the administration of PT determines whether DTH is enhanced or suppressed (243). PT (100 ng to 1 μg) administered one to twenty-one days after immunization results in the enhancement of the DTH response (218,245). This enhancement occurs at the same time as a decrease in T_S cells and the appearance of activated T cells (217,245). Furthermore, when PT is added to mice or to cells *in vitro* at the time of suppressor induction, the antigen-specific suppressor activity is decreased in a dose-dependent fashion without an increase in cell death (242). These results suggest that the abrogation of T_S effects by PT mediates the enhancement of DTH by the toxin. The restoration of DTH in unresponsive mouse cells by PT also occurs in mitomycin C-treated splenic T cells, indicating that cell division is not necessary for the effect (244). In contrast, a recent report (96) suggested that PT can prevent the induction of peripheral T cell unresponsiveness, though it does not affect established anergy. The mechanism responsible for these effects was not defined.

Enhancement of suppressor cell activity is also seen after administration of PT. Lyt 2^+ T lymphocytes are responsible for the PT-mediated, dose-dependent, cytokine-independent suppression of the cytotoxic T lymphocyte

response in mice (10). This effect was attributed to the selective proliferation of a T_S population. Huang (87) demonstrated, however, that the Con A-induced T_S activity of human lymphocytes is enhanced by PT, and that this enhancement occurs prior to the proliferation of $CD8^+$ cells. The effects of PT on DTH are therefore very similar to its effects on adjuvancy, and may be mediated through similar mechanisms. Though these effects are seen at higher doses of the toxin than are required for lymphocytosis, the fact that they occur in the absence of detectable cell proliferation is consistent with a mechanism involving the modification of G proteins by PT.

Purified PT is capable of increasing the production of IgE after immunization of animals with a specific antigen (158,239). The toxin also increases the expression of the low-affinity Fcε receptor on B cells (92) as well as the proportion of cells bearing IgE (161). Furthermore, IgE antibodies to PT are found in humans after immunization or disease (74). Many of these effects are the result of the PT-stimulated production, by T lymphocytes, of a glycosylating enzyme that converts an IgE binding factor to a stimulator of IgE production (92). As little as 0.1 ng of PT given at the time of immunization causes a hundred fold rise in IgE titres, though a recombinant PT with a genetically inactivated S1 subunit does not produce the increase in titres or enhanced IgE binding to cells (161). These results indicate that the IgE adjuvancy characteristic of PT is mediated through the ADP-ribosylation of specific G proteins.

The production of IL-2 by Con A-activated T lymphocytes is inhibited by submitogenic concentrations of PT (60,228). This is not due to competition for Con A receptors on cells by PT, since high concentrations of the toxin only slightly reduce the binding of Con A-FITC (60). Phospholipase C activation in Jurkat cells is not mediated through a PT-sensitive G protein (13,66). Further work is necessary to

determine the precise role of G proteins in signalling in this cell line.

It is clear from the preceding discussion that the effects of PT on T lymphocytes are extremely complex. The changes in nomenclature for describing T lymphocyte subsets add to the confusion, while changes in our understanding of the relative roles in the immune system of the human Th_1, Th_2, and $CD8^+$ cells and their mouse equivalents may require changes in the interpretation of some data. In order to avoid drawing inappropriate conclusions, for the most part I have used the original nomenclature for the T cell subsets and have not changed the interpretations regarding which subsets are involved in mediating the different effects of PT.

Some of the activities of PT described in this section could be explained by the differential activation of Th_1 and Th_2 cells and the cytokines they produce. Other activities are more likely the result of the direct interaction of PT with different T lymphocyte types. The data reviewed above suggest that PT can interact with all T cell subsets through one or more receptors or receptor complexes. Furthermore, the broad species specificity of PT action suggests that the T lymphocyte receptors for PT have been conserved through evolution. Candidates for the PT receptor on T cells that do not meet these criteria can therefore likely be eliminated.

The various effects of PT could be produced either through the binding of the toxin to a single receptor or receptor complex or through toxin binding to different receptors with different affinities. The first alternative is consistent with the commonly held idea that the high PT concentrations necessary for mitogenesis result from a requirement for extensive crosslinking of T cell-surface receptors. Ledbetter *et al.* (122) have shown that the crosslinking of many surface antigens causes activation of these cells, and that the extent of crosslinking affects the magnitude of the response. However, the fact that PT appears to cause a progressive disruption of T cell function with

increasing concentrations of the toxin could also be explained by differential affinities for PT of multiple cell-surface receptors. This is consistent with the idea that PT's dimer 1 and dimer 2 may interact with different receptors on eukaryotic cells. The elucidation of T lymphocyte receptors for PT is therefore necessary to develop an understanding of the role of PT in producing its effects on these cells.

E. Effects of PT on B lymphocytes.

PT treatment of a human B lymphocyte cell line inhibits the Con A-mediated capping and Ca^{2+} mobilization of these cells in a dose-dependent manner, with a half-maximal stimulation at 0.5 µg/ml of PT and a maximal effect at 1 µg/ml (63). An overnight treatment of these cells with the toxin is required for the full effect, consistent with the involvement of PT-sensitive G proteins. Similarly, PT partially inhibits the anti-immunoglobulin-stimulated proliferation of, and inositol phosphate formation in, murine splenic B cells (22). PT causes these effects at concentrations ranging from 5 to 500 ng/ml, again suggesting the involvement of a G protein. The fact that a 4 hour incubation period is necessary for this inhibition, as well as the fact that B lymphocyte proliferation in response to LPS or phorbol myristic acid (PMA) is not affected by PT treatment, supports this hypothesis. Human tonsillar B lymphocytes pretreated with 10 ng/ml of PT exhibit enhanced levels of *c-myc* RNA in response to stimulation with anti-IgM antibodies, while this concentration of the toxin has no effect on levels of *c-myc* RNA stimulated by anti-CD 20 antibodies (270). Increasing the concentration of PT to 50 ng/ml results in an inhibition of *c-myc* RNA levels after stimulation by anti-CD 20 antibodies. Two different signalling pathways for *c-myc* induction, with different sensitivities to PT, therefore exist in B lymphocytes. Actin condensation in human B lymphocytes in response to anti-IgM antibodies or *Staphylococcus aureus* Cowan I, but not phorbol

esters, is inhibited by PT in a dose dependent manner (142). A 3 hour pretreatment with a minimum PT concentration of 100 ng/ml is required for this effect. Finally, PT added at a concentration of 10 ng/ml inhibits the IL-1-induced immunoglobulin light chain synthesis in the pre-B cell line 70Z/3 (38). As with T lymphocytes, therefore, increasing concentrations of PT cause a progressive disruption of B cell function, presumably by uncoupling G proteins from different signalling pathways that require different G protein occupancy.

B cells are involved in the lymphocytosis stimulated by PT due to the release of these cells from lymphoid organs and the inability of these cells to home back to lymphoid organs (12,152,157,217). Concentrations of PT greater than 100 ng/ml are mitogenic for human peripheral B lymphocytes (109). The isolated toxin B oligomer is also capable of inducing mitogenesis, and this activity is correlated with increases in intracellular calcium due to the opening of a calcium channel (109). B lymphocytes therefore appear to respond to PT in ways similar to T lymphocytes. Though the receptor for PT on B cells is not known, it appears to be conserved during the process of B cell activation (109). It would be interesting to determine whether B and T cells have identical PT receptors or if these receptors are functionally homologous.

F. Effects of PT on monocytes and macrophages.

The effect of PT on macrophage and monocyte migration, chemotaxis, and elongation in response to stimulatory factors has recently been reviewed (157). Injection of PT into mice causes a monocytosis and inhibition of attractant-stimulated increase in peritoneal macrophages (139,140,253), with a concomitant reduction in macrophage elongation and random migration (20). Endotoxin activated mouse serum (EAMS)- and formyl-Met-Leu-Phe (fMLP)-stimulated chemotaxis in RAW 264 cells, a macrophage cell line, is inhibited in a dose-

dependent fashion by PT (14,138) with a similar time lag, temperature dependence, and concentration dependence as the PT-catalyzed ADP-ribosylation of a 41 kDa G protein. Chemotaxis of macrophages is inhibited 50% by 0.2 to 0.3 ng/ml of PT, while a concentration of 1 to 2 ng of PT is required for a similar inhibition of chemotaxis in RAW 264 cells (138). Increasing the toxin concentration to 20 ng/ml results in an 80% inhibition in chemotaxis and additionally causes a 25% to 35% inhibition of Fc-mediated phagocytosis in macrophages (138). Finally, PT can act as a chemoattractant for human blood monocytes at concentrations ranging from 100 ng/ml to 10 µg/ml (182), an activity that may be due to the lectin-like binding of the toxin at higher concentrations (138).

Monocytes and macrophages from mice and humans produce IL-1 in response to stimulation with LPS. Low doses of PT (< 1 µg/ml) suppress IL-1 production by these cells, while higher doses (2.5 to 10 µg/ml) enhance IL-1 production (260). In addition, PT reduces the LPS-stimulated IL-1 production in the macrophage cell line $P388D_1$, exhibiting a 50% inhibition after a 4 hour treatment of cells with 0.3 ng/ml of the toxin (94). This concentration of the toxin also causes the ADP-ribosylation of 50% of the G_i molecules in $P388D_1$ membranes, suggesting that G proteins are involved.

Interestingly, 1 µg/ml of PT alone can stimulate IL-1 production by human monocytes through a different pathway than the one activated by LPS (246). Specific inhibition of protein kinase C activation and calmodulin-dependent phosphorylation, as well as chelation of intracellular calcium, suppress this PT-dependent activity. Similar concentrations of PT block the leukotriene B_4 and formylpeptide stimulation of O_2^- production and phosphatidyl-inositol hydrolysis in macrophages (84), and also decrease the LPS-stimulated prostaglandin E_2, leukotriene C_4, and eicosanoid release from macrophages (137). These effects are

all thought to result from the PT-mediated ADP-ribosylation of G proteins.

Griese *et al.* reported that PT reduces both basal and prostaglandin E_1-stimulated cAMP levels in a dose-dependent fashion in human mononuclear leukocytes (64). The minimum concentration of PT required to produce this effect was 0.01 ng/ml, while the concentration producing the half-maximal inhibition was in the range of 1 to 10 ng/ml, consistent with the involvement of specific G proteins. In contrast, Maisel *et al.* (135) found that PT had no effect on the basal or prostaglandin E_1-stimulated cAMP levels produced by monocytes from the blood of normal humans, though PT treatment did enhance cAMP production in monocytes from the blood of patients with congestive heart failure. Since whole blood was treated with PT in this latter study, the effects of PT on monocytes could have resulted in part from the activation of, and cytokine production by, other leukocytes in the blood.

Taken together, the data indicate that PT can bind to monocytes and macrophages and influence cellular signalling events. The minimum concentrations of PT required to produce these effects is similar to the concentrations required to produce effects in lymphocytes, suggesting there may be similarities at the level of toxin receptors.

G. Effects of PT on mast cells and basophils.

Histamine release from rat mast cells stimulated by compound 48/80 or binding of IgE receptors is inhibited *in vivo* and *in vitro* by PT (164). As with other cell types, this inhibition is time and concentration dependent, occurring at a minimum dose of 0.1 to 0.3 µg/rat or approximately 0.1 ng/ml for isolated cells (half-maximal effect at 0.25 ng/ml of PT) (164,165). Changes in cAMP levels are not associated with this inhibition, though toxin pretreatment does decrease the breakdown of inositol-4,5-bisphosphate, inositol-1,4,5-triphosphate generation, ^{32}P incorporation into phosphatidylcholine, ^{32}P incorporation into phosphatidic acid, and

arachidonic acid release (164,166). The extent of ADP-ribosylation of G_i proteins correlates with the effects seen, suggesting that G proteins are involved (155). Moreover, the observation that the activation of mast cells can be achieved through Ca^{2+}-permeable channels regulated by a PT-sensitive G protein (116), or by IgE-dependent mechanisms that do not involve calcium channels, suggests there may be more than one class of G protein modified by the toxin in mast cells. It is clear, however, that PT can bind to and cause effects in these cells at concentrations found to be effective in other cell types.

Pretreatment of rat basophilic leukemia cells with 100 ng/ml of PT decreases the antigen-stimulated $^{86}Rb^+$ efflux through a K^+ channel in these cells (118). This concentration of PT has no effect on antigen-stimulated Ca^{2+} influx in these cells. High concentrations of PT (1 μg/ml) inhibit C5a- and fMLP-stimulated histamine and leukotriene B_4 release from human basophils, though, in contrast to rat mast cells, this toxin concentration has no effect on histamine release in response to anti-IgE (267).

Finally, PT reduces the proportion of IC2 cells, a mast cell progenitor line, that proceed to the G2 phase of the cell cycle and subsequently engage in DNA synthesis in response to stimulation by cytokines (112). The inhibition of DNA synthesis is detectable after treatment with as little as 0.01 ng/ml of the toxin for 21 hours in the presence of the stimulating cytokine, and the maximal effect is seen when 1 ng/ml of PT or more is used. Cell cycle analysis of the affected cells reveals that PT increases the fraction of cells in G1, though it does not inhibit the binding of cytokines to cell surface receptors. A 41 kDa G protein is fully ADP-ribosylated under conditions that completely inhibit DNA syntheses.

H. Effects of PT on neutrophils and eosinophils.

Many of the investigations into the effect of PT on granulocytes have concentrated on the dissection of G protein involvement in a multitude of signalling pathways, and much of this information has been catalogued in recent reviews (19,157). The effects of PT on neutrophils include the following: inhibition of granule enzyme secretion, chemotaxis, O_2^- generation, aggregation, CR 3 expression, and arachidonic acid production in response to a variety of stimuli (19,26). PT also inhibits phagocytosis by neutrophils of IgG-opsonized, but not serum opsonized, bacteria (80). Addition of the purified PT B oligomer does not affect these activities (76,184). Most data indicate that PT ADP-ribosylates a G protein coupling phospholipase C to cell-surface receptors (19,221), though a G protein responsible for calcium mobilization may also be enzymatically modified by PT (42,183). The Con A-induced capping of neutrophils, which is inhibited by larger doses (15 µg/ml) of PT, is accompanied by the inhibition of calcium mobilization stimulated by fMLP, platelet activating factor, and Con A (119). PT can cause an influx of calcium into neutrophils after stimulation with fMLP, suggesting that the toxin either possesses or regulates a calcium ionophore activity in these cells (226). Finally, the inhibition of fMLP-stimulated leukotriene B_4 production by PT (77) is partly due to a decrease in the number of fMLP receptors on the surface of these cells (76), indicating that PT can generate changes in cells not directly related to the ADP-ribosylation of G proteins.

Neutrophil accumulation in peripheral sites of inflammation (88,226) and the estradiol-stimulated eosinophil accumulation in the uterus (132) are strongly inhibited by PT due to the effects on neutrophils and eosinophils themselves (178,132). Though neutrophils do not persist and accumulate in the blood in a manner similar to lymphocytes, they do appear in the spleen (226). At the same time, there is an

increase in the total number of neutrophils due to the stimulation of granulopoiesis in the bone marrow (88). This, however, may be due to the stimulation by PT of bone marrow stromal cells or lymphocytes, not neutrophils themselves.

Low concentrations of PT are sufficient to produce most effects of the toxin, and these effects are time and concentration dependent (23). Furthermore, different neutrophil signalling pathways disrupted by PT are sensitive to different concentrations of the toxin (184,226). For example, the concentration of PT required for half-maximal inhibition of arachidonate release is 0.2 ng/ml, and the concentration necessary for half-maximal inhibition of O_2^- generation is 0.5 ng/ml, consistent with a concentration of 0.5 ng/ml for the ADP-ribosylation of 50% of the cellular G proteins (183). Half-maximal inhibition by PT of leukotriene B4 generation, however, occurs at toxin concentrations of 100 ng/ml (76), five hundred times the amount required for inhibition of arachidonate release. PT treatment regimes that completely inhibit oxidant production in neutrophils only partially inhibit the fMLP-induced actin-associated right angle light scatter response, indicating that the right angle light scatter response is induced by fewer occupied receptors than oxidant production in these cells (184). A practical consequence of this observation is that different responses produced in cells by PT treatment need not be the result of differences in PT binding to cell surface receptors. This makes it more difficult to differentiate the actions of PT due to binding versus those due to G protein modification without the use of the PT B oligomer as a control for binding effects.

The PT receptors on neutrophils, HL-60 cells, and eosinophils have not yet been identified. PT affects neutrophils from rabbits (19), humans (220,221), guinea pigs (183), and mice (88,226), suggesting that the receptor is at least functionally homologous in all these species.

I. Effects of PT on other cells.

As can be seen from Tables 1 and 2, PT has effects on many different cell types from a number of different organisms. These tables likely contain only a fraction of the information available, since there were 591 references to PT cited in MedLine in 1991 alone, most of which deal with the toxin's effects on eukaryotic cells. It is apparent from these tables, however, that PT is capable of binding to, and modifying the activity of, a large number of cells and cell lines. The actions of PT on adipocytes, islet β cells, fat cells, heart muscle cells, C6 glioma cells, and endothelial cells have been the subject of numerous recent reviews (49,53,187,256,257,265; see also Tables 1 and 2). Evidence that PT can modify the function of signalling pathways in nerve cells is also accumulating, and has recently been reviewed (105,188). In fact, most cells and cell lines seem to be responsive to PT at some concentration, leaving one to ask instead whether there are any cells that do not produce receptors for the toxin. This may also be the case *in vivo*, given the varied metabolic and biochemical changes produced in patients with pertussis (53,187,263,265,266). In general, the concentrations of PT required for the modification of cellular function are very low, consistent with the involvement of G proteins and suggestive of the presence of relatively high affinity receptors for PT. It has recently been reported, however, that a submitogenic concentration of PT's B oligomer (100 ng/ml) inhibits the IL-1-induced production of IL-2 mRNA and protein in the EL4 thymoma line and also inhibits the IL-1-stimulated prostaglandin E_2 synthesis in human gingival fibroblasts (180). It remains possible that binding of the PT B oligomer to various cell types may actually produce some of the effects that have been attributed by circumstantial evidence to the enzymatic action of the toxin.

J. Rationale for experimentation.

The information reviewed above indicates that PT is an extremely interesting molecule that is important in disease, in whole cell, acellular, and genetically modified vaccines, and in pharmacological research. In order to fully understand the functions of the toxin and to correctly interpret its role in the modification of cell signalling pathways, we need to identify the plasma membrane receptors for PT on eukaryotic cells and more fully define the effects of toxin binding. T lymphocytes and Jurkat cells provide an excellent model system to begin these investigations, since a great deal is currently known about the modulation of the functions of these cells. My first priority was therefore the identification of T lymphocyte and Jurkat cell receptors for PT. After this was accomplished, I set out to characterize these receptors and to identify receptors for PT on other eukaryotic cells.

Table I.1. Some *in vitro* activities of PT on cells and cell lines.[1]

Cell type	Activity	Concentration[2] for half-maximal effect (ng/ml)	Minimum[1] conc. for effect (ng/ml)	References
HUMAN				
T lymphocytes	mitogenesis	ND[3]	100-500	(134,233)
	chemotaxis to IL8	18	1	(271)
T cell lines	inhibition of invasion through fibroblast monolayers	0.006	0.001	(259)
B lymphocytes	mitogenesis	~250	64	(109)
	potentiation of response to LMW-BCGF	ND	25	(109)
B cell lines	calcium mobilization	500	ND	(63)
monocytes/ macrophages	decrease of PGE_1-stimulated cAMP levels	1-10	0.01	(64)
	inhibition of eicosanoid release	~200	10	(137)
macrophage cell lines	inhibition of LPS-induced responses	0.5	0.1	(94)
neutrophils	increase in myelo-peroxidase release	ND	5	(226)

Table I.1. (continued)

Cell type	Activity	Concentration[2] for half-maximal effect (ng/ml)	Minimum[1] conc. for effect (ng/ml)	References
HL-60 cells	inhibition of uridine transport	ND	1	(222)
	inhibition of inositol phosphate accumulation	ND	100	(42)
fibroblasts	increased bradykinin receptor number	ND	50	(47)
HEL cells	inhibition of prostaglandin-desensitization of cAMP stimulation	ND	50	(11)
platelets	activation	ND	5000	(16)
MOUSE				
T lymphocytes	lymphocytosis	0.2-4.5	0.1	(225,236)
	mitogenesis	250-500	250	(111,217)
	IL 2 production	1.7	~0.5	(202)
	migration to PT	ND	10	(251)
B lymphocytes	inhibition of anti-Ig-stimulated proliferation	ND	5	(22)
neutrophils	inhibition of extravasation	ND	5	(226)
BALB/c3T3	inhibition of DNA synthesis stimulated by EGF, insulin	ND	100	(43)

Table I.1. (continued)

Cell type	Activity	Concentration[2] for half-maximal effect (ng/ml)	Minimum[1] conc. for effect (ng/ml)	References
macrophages	inhibition of migration	ND	1	(139)
IC2 mast cell line	inhibition of growth-factor induced DNA synthesis	~0.02-0.05	0.01	(112)
RAT				
C6 glioma cells	potentiation of adenlyate cyclase activity	~30-50	0.001-10	(101,104,177)
adipocytes	glycerol release	~1-20	~0.3	(177,214)
	insulin release	0.1	0.001	(102,177)
	increase in glucose oxidation	(~30µg/ml)	(~10µg/ml)	(177)
islet cells	enhanced insulin release in response to glucose	0.1	0.001	(102,103)
	reversal of inhibition of insulin release	0.1	0.001	(102)
astrocytes	inhibition of prostaglandin synthesis	ND	50	(58)
mesangial (kidney) cells	inhibition of topoisomerase I activation	~20	1	(167)

Table I.1. (continued)

Cell type	Activity	Concentration[2] for half-maximal effect (ng/ml)	Minimum[1] conc. for effect (ng/ml)	References
neuronal and glial cells	inhibition of norepinehrine-stimulated inositol phosphate formation	7	0.1-1	(272)
heart cells	enhanced cAMP accumulation	80	10-50	(73)
mast cells	inhibition of histamine release	0.25	ND	(164,175)
	inhibition of arachidonate release	0.3	0.1	(165,166)
FAO (hepatoma)	inhibition of insulin-stimulated insulin receptor autophosphorylation	1000	ND	(162)
Guinea pig				
neutrophils	inhibition of arachidonate release (agonist-stimulated)	0.2	ND	(183)
	inhibition of agonist-stimulated O_2^- generation	0.5	ND	(183)
	inhibition of leukotriene B_4 generation	100	ND	(76)

Table I.1. (continued)

Cell type	Activity	Concentration[2] for half-maximal effect (ng/ml)	Minimum[1] conc. for effect (ng/ml)	References
uterine artery (endothelial cells)	attenuation of response to neuropeptide Y	ND	1000	(147)
	attenuation of contractions in response to prostaglandin $F_{2\alpha}$	ND	250	(147)
OTHER				
CHO cells	clustered growth pattern	~0.35	0.03-0.12	(79,273)
LEC 2 cells (CHO lacking sialic acid residues)	clustered growth pattern	ND	250	(273)
LEC 8 cells	clustered growth pattern	ND	31	(273)
	modification of G proteins	ND	>10	(273)
(CHO lacking sialic acid and galactose residues)				
NIH3T3	inhibition of growth rate	ND	1	(81)
bovine endothelial cells	inhibition of adenosine-stimulated proliferation	ND	1	(141)
cuckoo wrasse melanophores	inhibition of induced pigment aggregation	~0.001	(10^{-16} g/ml)	(3,97)

[1] Adapted from Munoz (156). In some cases, cells were treated *in vitro* with PT, then injected into animals in order to analyze the effects of the toxin. Because of the vast amount of information available in the literature, this should not be considered a complete catalog of PT's activities.

[2] These values were generally the lowest found in the literature for a particular effect. The concentration required to produce any particular effect could vary with the lot of PT used as well as the time of incubation. Longer incubation times generally give effects at lower concentrations of PT. In some instances (marked with ~), I have estimated a 50% effective dose or dose range from published data.

[3] ND means not determined

Table I.2. PT effects on G protein-mediated signalling pathways.

Activity	Dose used	References
Human		
cultured foreskin fibroblasts - enhancement of bradykinin-stimulated arachidonic acid release	50 ng/ml	(47)
astrocytoma cells - reversal of the inhibition by adenosine of histamine-stimulated accumulation of inositol phosphates	100 ng/ml	(163)
astrocytoma cells - potentiation of the cAMP response to adenosine and adenosine analogs	200 ng/ml	(2)
eosinophils - prevention of eosinophil peroxidase exocytosis, intracellular calcium changes, and shape changes	2000 ng/ml	(106)
Mouse		
histamine sensitization	0.5 ng/mouse	(158)
increase in capillary permeability	0.5 ng/mouse	(158)
leukocytosis and lymphocytosis	8-40 ng/mouse	(158)
enhancement of insulin secretion	2 ng/mouse	(158)
immunopotentiation of IgE production	0.1 ng/mouse	(158)

Table I.2. (continued)

Activity	Dose used	References
immunopotentiation of IgG_1 production	10 ng/mouse	(158)
anaphylaxis	9.5 ng/mouse	(158)
lethality	546 ng/mouse	(158)
decrease in morphine-induced analgesia and toxicity in mouse neurons	200 ng/mouse	(131)
increase in locomotor activity, decrease in exploratory head dipping, decrease in the hypothermic response to alcohol	500 ng/mouse	(44)
elimination of opioid-induced place preference	500 ng/mouse	(237)
Rat		
blunting of the baroreceptor response	80 fmol into brain (nucleus tractus solitarus)	(52)
prevention of apomorphine and oxytocin induced penile erection and yawning	0.5 µg/rat (paraventricular nucleus)	(227)
increase in cocaine-stimulated motor activity	1 µg/rat	(230)
attenuation of morphine withdrawal syndrome	1 µg/rat	(133)
neurons - inhibition of G proteins coupled to D1 receptors in the neurons of the rat brain	500 ng/brain	(235)

Table I.2. (continued)

Activity	Dose used	References
neurons - decrease of the rhythmical bursting activity	2 µg/rat	(45)
neurons - inhibition of the neuropeptide Y reduction of p-[^{3}H]aminoclonidine binding affinity	10 µg/rat	(262)
neurons - attenuation of the effects of adenosine A_1	1.5 µg/rat	(50,232)
neurons - decrease in the levels of substance P and serotonin in the brain and spinal cord	500 ng/rat	(57)
hepatocytes - elimination of EGF-induced tyrosine phosphorylation of PLC-γ and Ca^+ mobilization	10 µg/100 g	(275)
cultured astrocytes - inhibition of (stimulated) prostaglandin D_2 synthesis and arachidonic acid release	50 ng/ml	(58)
cultured arterioles - inhibition of the neuropeptide Y potentiation of stimulated arteriole contraction	300 ng/ml	(4)
isolated islets - enhancement of insulin secretion	300 ng/ml	(35)
isolated fat cells - increase in cAMP accumulation and uncoupling of inhibition by insulin, adenosine analogs, prostaglandin E_2, glycosyl inositol phosphate	10 µg/ml	(136)

Table I.2. (continued)

Activity	Dose used	References
Rabbit		
endothelium - inhibition of endothelium contractions produced by agonist stimulation	5 µg/kg body wt.	(83)
Cow		
arteries - decrease in relaxation of arteries and elevation of cGMP in arteries in response to vasodilators	100 ng/ml	(127)
heart endothelial cells - blocking of adenosine-stimulated proliferation	1 ng/ml	(141)
Opossum		
kidney cells in culture - enhancement of stimulated cAMP levels	100 ng/ml	(55)
kidney cells - attenuation of parathyroid hormone inhibition of Na/Pi cotransport and inositol phosphate generation; enhanced parathyroid hormone-dependent cAMP generation	5 ng/ml	(198)

Table I.2. (continued)

Activity	Dose used	References
Hamster		
CCL39 fibroblasts - inhibition of thrombin stimulated activation of mitogen-activated protein kinase	50 ng/ml	(117)
Frog		
adrenocortical cells - elimination of the inhibitory action of dopamine on corticosteroid production	1 µg/ml	(146)
Snail		
inhibition of avoidance behaviours	100 ng/snail	(276)

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PREFACE TO CHAPTER II

To characterize receptors for PT, we first needed to identify cell-surface proteins capable of specifically binding to the toxin. The second step would then be to demonstrate whether toxin binding to these particular proteins was capable of directly producing a detectable biological effect in the intoxicated cell. Though we have identified PT-binding proteins on several cell types, we have not yet determined that the known biological effects of PT on these cells are mediated through toxin binding to these proteins. In this thesis, I will use the word receptor in a non-pharmacological sense to indicate PT-binding proteins that may produce cellular effects.

Several methods are available for the identification of receptors on cells (1). One useful technique is the affinity-isolation of receptor proteins using immobilized lectins, antibodies specific for either the receptor or ligand, or the ligand itself. Immunoprecipitation of receptors and receptor complexes with specific antibodies may be considered a variation on the affinity isolation technique. Membranes solubilized using detergents are incubated with the immobilized reagents and non-adsorbed cellular components are removed by washing. Absorbed receptors are subsequently eluted either by changing the pH or ionic strength of the buffer, or by competition for binding using competitive inhibitors. Because affinity columns may retain proteins that bind through both low and high affinity interactions, this technique can lead to the identification of binding proteins that may have no biologically significant receptor activity. The disruption of the natural membrane architecture with detergents may also expose binding proteins that would normally be unavailable for interaction with the ligand under investigation. Finally, detergent solubilization of membranes may facilitate the action of intrinsic proteases, leading to degradation of the receptor (1). Despite these potential

drawbacks, affinity isolation techniques remain extremely useful for receptor identification. The potential for obtaining undenatured proteins and intact protein complexes using appropriate conditions represent major advantages of these techniques.

Receptors can also be identified by separating solubilized membrane proteins using SDS-PAGE, transferring the proteins to nitrocellulose, and probing the resulting blots with either radiolabeled ligand or unlabeled ligand and a ligand-specific antibody-enzyme conjugate. In addition to the drawbacks discussed above for affinity isolation techniques, proteins obtained using this procedure are often partially or totally denatured, resulting in the loss of binding sites or the appearance of binding activity not normally present in the undenatured protein. Despite these problems, however, Brennan *et al.* have used this technique to identify a 165 kDa PT-binding glycoprotein on CHO cells (3). Their success may result partly from the inability of detergents to denature specific oligosaccharide sequence determinants necessary for toxin binding. Because of this, blotting procedures may be extremely useful for the identification of glycoprotein receptors for lectins. Protein denaturation may still affect the ability of the ligand to recognize its receptor, though, since differences in protein structure appear to affect the availability or spatial presentation of the oligosaccharide receptor sequence (2). Similarly, changes in ceramide structure affect the conformation of sugars in lactosylceramide (4). The resulting confomers bind different bacterial species carrying lectin-like adhesins with different specificities.

Crosslinking and photoaffinity labeling of receptors or receptor-ligand complexes provide alternate methods for identifying receptors (1,5). Because ligands are allowed to interact with intact cells having undisturbed plasma membranes, the ligand is more likely to interact only with biologically relevant cell-surface molecules. Cross-linking

agents may be added to cells which already have their receptors occupied by ligand, covalently linking receptor and ligand. When monomeric, low molecular weight peptide ligands are used, the apparent molecular weight of the receptor can usually be determined by subtracting the known weight of the peptide ligand (eg EGF or insulin) from the apparent molecular weight of the ligand-receptor complex in SDS-PAGE gels. The analysis is facilitated by the fact that receptors are usually much larger than their ligands. Interpretation of these data is more difficult when multimeric or high molecular weight ligands such as PT are used. The use of cleavable, iodinatable photoaffinity labeling agents that leave only a small radioactive tag on the crosslinked receptor circumvents this problem (see Figure III.2 for an illustration of the technique). Crosslinking techniques are not without their own specific problems, however. Factors such as the length of the crosslinking agent and its distribution on the ligand may make productive interactions with receptors unlikely or impossible, and the covalent modification of the ligand with photoaffinity cross-linking agents could result in the loss of receptor-binding activity (5). Irrelevant proteins proximal to the receptor could conceivably be labeled as a result of these geometrical considerations, though the use of different crosslinking agents for these investigations should reduce the chances of drawing inappropriate conclusions from these types of studies.

When we began our investigations, it was apparent that no single technique would be adequate for unambiguously identifying receptors for PT on eukaryotic cells. We therefore decided to use a combination of several different procedures in our studies.

At the time the study reported in Chapter II was initiated, the oligosaccharide sequences required for PT binding to fetuin had been recently characterized (2; see section I.C.1). The identification of a 165 kDa glycoprotein

as a PT receptor on CHO cells (3) suggested that eukaryotic cell receptors for the toxin may carry binding determinants similar to those on glycoproteins such as fetuin, and indicated that the information obtained from studies of serum glycoproteins could contribute to an understanding of toxin interaction with these eukaryotic receptors.

The characterization of PT binding to glycoproteins and goose erythrocytes presented in this chapter was partially completed at the time I began my studies. This area of study therefore offered an excellent model system with which to begin investigations into PT receptors on eukaryotic cells. As can be seen in Figure II.2 (below), affinity chromatography of iodinated goose erythrocyte proteins using PT-agarose identified several potential receptors for PT on these cells. We felt it was unlikely that all of the proteins identified in these studies would act as receptors for PT on the intact erythrocyte, and that many of these proteins may be binding to PT-agarose through electrostatic interactions. I therefore sought to identify procedural modifications that would eliminate non-specific interactions and identify goose erythrocyte surface proteins that bind PT with high affinity.

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Chapter II

LECTINLIKE PROPERTIES OF PERTUSSIS TOXIN: CHARACTERIZATION OF THE BINDING PROPERTIES OF PERTUSSIS TOXIN WITH GLYCOPROTEINS AND GOOSE ERYTHROCYTES AND COMPARISON WITH WHEAT GERM AGGLUTININ[1]

A. INTRODUCTION

The structure of pertussis toxin (PT) is well defined, but the role of its structure in pathophysiology is incompletely understood. While most of the biological effects of PT may be related to the ADP-ribosyltransferase activity of the S_1 (enzymatic) subunit (13), the mitogenic activity of PT is apparently the result of the interaction of the basal components with lymphocyte receptors (12, 14). The lectin-like activity of the PT B oligomer may also contribute to the attachment of *B. pertussis* organisms to respiratory epithelial cells (19). Accordingly, it is important to identify and characterize receptors for pertussis toxin in order to fully understand its activity in the disease process.

In our previous report (3) we identified important carbohydrate groups involved in the lectin-like interaction of PT with the glycoprotein fetuin. Our studies suggested that PT binds to similar sugar sequences as the plant lectin, wheat germ agglutinin (WGA). However, we also showed that sugar sequence alone does not predict the ability of PT to

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[1] A version of this chapter has been published. Tyrrell, G. J., M. S. Peppler, R. A. Bonnah, **C. G. Clark**, P. Chong, and G. D. Armstrong. 1989. Infect. Immun. **57**:1854-1857. R. A. Bonnah performed the binding inhibition studies (Figure 1), and G. J. Tyrrell completed the initial characterization of goose erythrocyte-protein binding to PT-, WGA-, CT-, and fetuin-agarose affinity columns (Figure 2). I contributed the analysis of goose erythrocyte-protein interaction with PT-, I-PT-, histone-, and fetuin-agarose columns after washing with buffer containing 1 M NaCl (Figure 3).

bind to glycoproteins. For example, α1 acid glycoprotein contains fetuin-like sugar sequences (11) but bound less well than fetuin to PT. Bhavanandan and Katlic (5) reported a similar disparity between sugar sequence and binding affinity in the WGA system. Therefore, it appeared that PT and WGA receptor activity may depend on glycoprotein elements in addition to sugar sequences in the glycan units. In the present report, we explore the extent of functional homology between these two lectins by comparing the abilities of various glycoproteins and potential receptors to bind to PT and WGA.

B. MATERIALS AND METHODS

1. Materials.

Goose erythrocytes (diluted 50:50 in Alsever's solution) were obtained from Gibmar laboratories, Ardrossan, Alberta. PT was kindly donated by Connaught laboratories, Willowdale, Ontario. Cholera toxin (CT) B subunit was obtained from List Biological Laboratories Inc., Campbell, California. Activated CH-Sepharose and wheat germ agglutinin (WGA)-agarose were obtained from Pharmacia. Fetuin-agarose, WGA-agarose, and histones (type VII-S from calf thymus) were obtained from Sigma.

2. Binding inhibition assays.

PT and WGA binding inhibition assays were performed in fetuin-coated 12 x 75 mm polystyrene culture tubes. Prior to the fetuin coating procedure dust particles were removed from the tubes using a stream of dry nitrogen gas. Fetuin was dissolved at a concentration of 50 µg/mL in 50 mM sodium phosphate buffer (pH 6.8) containing 5 mM $MgCl_2$ and 15 mM sodium azide as the bacteriostatic agent. Next, 100 µL of the fetuin solution was carefully placed in the bottom of each polystyrene tube. The tubes were incubated overnight

(uncapped) at room temperature in a water saturated environment to prevent evaporation. The solution containing unbound fetuin was then removed from the tubes by aspiration and replaced with 0.5 mL of 5% BSA dissolved in sodium phosphate buffered (pH 7.2) physiological saline (PBS). This solution was incubated in the tubes for 4 h at room temperature to allow BSA to bind to all the protein attachment sites not occupied by fetuin. The BSA solution was then removed by aspiration and the tubes were washed with three successive 0.5 mL portions of PBS. The tubes were shaken with a Vortex mixer and incubated for 10 min between each addition of PBS wash solution.

Biologically active, ^{125}I-labeled PT was prepared by the procedure described earlier (4). WGA was iodinated by the Iodo Gen procedure (7) described by Armstrong *et al.* (1) with the exception that the reaction was carried out in the presence of 0.4 M N-acetylglucosamine (GlcNAc) to prevent iodination damage to the WGA binding sites. The number of ^{125}Iodine counts incorporated into pertussis toxin or wheat germ agglutinin was determined by TCA precipitation as described previously (4). Incorporated iodine counts were routinely in excess of 80% of the total counts remaining in the preparations after removing free iodine from the reaction mixtures. The unlabeled inhibitor solutions were prepared in PBS containing 0.1% BSA. One hundred µL of each inhibitor solution was added to the fetuin-coated polystyrene tubes prior to adding iodinated PT or WGA (5-10 µL). The final concentration of PT or WGA varied slightly from experiment to experiment but was approximately 1×10^{-9} M. The tubes were incubated overnight at 4°C in water saturated environment and unbound counts were removed by washing the tubes with 4 successive 0.5 mL portions of ice chilled PBS containing 0.1% BSA. Bound counts were measured in an LKB Rack Gamma model 1270 gamma counter. Background binding was determined in polystyrene tubes coated with BSA alone and the maximum binding in each experiment was measured in tubes containing

no inhibitor. Background binding was routinely 10% or less of the maximum binding. The concentration of inhibitor which produced a 50% reduction in binding (maximum counts bound minus the background counts) was calculated from graphical analysis of the data.

3. Iodination of surface-exposed erythrocyte proteins.

Two hundred µL of goose erythrocytes was suspended in 10 mL of PBS and centrifuged for 5 min at 500 x g in 15 mL conical centrifuge tubes. The erythrocyte cell pellet was washed by three cycles of centrifugation at 500 x g for 10 min in 10 mL PBS. The final erythrocyte pellet was suspended in 100 µL of PBS (2×10^8 cells/mL). The erythrocytes were then placed into glass 12 x 75 mm culture tubes which had been coated with 40 µg of Iodo-Gen. Approximately 5 MBq of Na[^{125}I] was added to the cell suspensions to initiate the iodination reaction. After 5 min, 100 µL of cysteine (1 mg/mL in PBS) was added to stop the reaction and the iodinated cells were transferred from the Iodo-Gen-coated tubes to 10 mL of PBS in 15 mL plastic conical centrifuge tubes. The cells were then washed by three cycles of centrifugation at 500 x g for 10 min in 10 mL PBS to remove unreacted [^{125}I].

4. Preparation of Sepharose-immobilized pertussis toxin, cholera toxin, and histone.

Immediately before use 0.33 mg of activated CH-Sepharose was hydrated and washed with 1 mM HCl on a sintered glass filter. PT (1 mg) was dissolved in 3.33 mL of 10 mM potassium phosphate (pH 7.5) containing 0.5 M NaCl. The lyophilized CT B subunit preparation (1 mg) was dissolved in 3.33 mL of distilled water and dialysed against pH 7.5 potassium phosphate-0.5 M NaCl buffer to remove the Tris salts added by the supplier. The PT and CT solutions were sonicated in a Branson model B-220 ultrasonic cleaner for 20 sec to break up aggregates and then transferred to 5 mL screw-cap vials which had been siliconized with a 5% solution of

dimethyldichlorosilane in chloroform. Next, the moist, HCl-washed CH-Sepharose was added to the vials and the mixtures were incubated on an slowly turning end-over-end rotator for 2 h at room temperature. The toxin-agarose suspensions were then transferred to sintered glass filters and washed with 50 mL of 50 mM Tris-HCl (pH 8.0) containing 0.5 M NaCl. The pH of the Tris-NaCl filtrate solutions was neutralized with HCl and the amount of PT or CT present was determined by measuring the optical density at a wavelength of 280 nm. Alternately, PT hemagglutinating activity in the Tris-NaCl filtrate solutions was determined by the hemagglutination assay using chymotrypsin-treated goose erythrocytes (4). The difference between the total amount of pertussis or cholera toxin added to the CH-Sepharose and the amount recovered in the Tris-NaCl wash solution allowed us to estimate the amount of PT or CT bound to the agarose. The derivatized agarose preparations were then suspended in 3 mL of the pH 8.0 Tris washing solution and incubated for an additional 60 min to inactivate the remaining reactive ester groups. Next, the preparations were washed with 50 mL of pH 8.0 Tris washing solution followed by 50 mL of 50 mM sodium acetate (pH 4.0) containing 0.5 M NaCl. Finally, the preparations were washed with 50 mL of PBS and suspended in an equal volume (approximately 1 mL) of PBS containing 0.1% sodium azide. Histone-agarose (1 mg histone/mL of gel) was prepared in the same manner. Iodinated PT-agarose was obtained by exposing a portion of the PT-agarose preparation to unradioactive NaI (40 μM) in the presence of chloramine T (7 mM) for 30 min. After iodination the PT-agarose was washed extensively with PBS to remove chloramine T and unreacted iodine. The iodination procedure was repeated three times. All of the agarose preparations were stored at 4°C until use.

5. Goose erythrocyte receptor affinity isolation procedure.

The surface labeled erythrocytes were sedimented by centrifugation as described above and suspended in 0.5 mL of 50 mM Tris-HCl (pH 8.1) containing 0.1% Triton X-100, 0.5 mg aprotinin, 0.5 mg α2 macroglobulin, 10 µg phenylmethylsulfonyl fluoride, 5 mM $CaCl_2$ and 5 mM $MgCl_2$. The cells were incubated in this solution for 30 min at 37°C to lyse the cells and extract membrane-associated proteins. The Triton solutions were then centrifuged at 100,000 x g at 4°C for 60 min to sediment the insoluble cellular components. The number of ^{125}I counts present in the resulting supernatant solution was determined in the gamma counter and the sedimented material was discarded. At least 75% of the total counts were present in the supernatant solutions. Two hundred µL of pertussis toxin-agarose, cholera toxin-agarose, wheat germ agglutinin-agarose, histone-agarose, or fetuin-agarose gel suspension was added to glass wool-plugged Pasteur pipets. The gels were then washed with 2 to 3 mL of PBS to remove the azide. The bed volume of the resulting affinity chromatography columns was approximately 100 µL.

The affinity chromatography procedure was performed at room temperature. 0.5 mL of PBS containing 1% BSA was passed through the affinity columns prior to adding the Triton-solubilized erythrocyte solutions. Next, 100 µL of surface iodinated erythrocyte solution (containing approximately 3 x 10^4 to 6 x 10^4 cpm) was added to each column. In any one experiment the number of counts added to each column was identical. No further additions were made to the columns for 15 min to allow the solubilized receptors adequate time to bind to the immobilized proteins. The columns were then washed with 4 mL of 50 mM Tris-HCl (pH 7.2) containing 110 mM NaCl, 5 mM $CaCl_2$, 5 mM $MgCl_2$, and 0.02% Triton X-100 to remove unbound iodinated material. 0.5 mL fractions were collected during the washing procedure and monitored in the gamma counter until the counts coming off the columns remained

essentially constant. The final 0.5 mL of washing buffer was retained to be analyzed by discontinuous SDS polyacrylamide gel electrophoresis (SDS-PAGE). Bound receptors were sequentially eluted with: 1) 0.4 M GlcNAc in wash buffer, and 2) 50 mM diethanolamine (pH 11.5) containing 0.15 M NaCl. Elution was performed by applying 100 µL of the eluting solution to the columns. The flow was then stopped for 15 min to allow the release of bound receptors from the affinity matrices. The columns were washed with 400 µL of washing buffer prior to the addition of each eluting solution. After the final (diethanolamine) elution step, the columns were washed with approximately 10 mL of PBS and stored at 4°C in the presence of 0.1% sodium azide in PBS to inhibit bacterial growth. If properly cared for, the columns could be used several times before losing their binding activity.

6. SDS-PAGE analysis of affinity isolated erythrocyte receptors.

The eluted receptors were dialysed overnight at 4°C against 0.1% SDS in distilled water and lyophilized using a Savant model SVC100H Speed Vac concentrator. The dialysis step was necessary to remove GlcNAc and salt which would interfere with the SDS-PAGE analysis. The loss of ^{125}I counts during dialysis was less than 5%. The dried receptors were then dissolved in 50 µL of Laemmli sample buffer (8) and analyzed in the presence of β mercaptoethanol by SDS-PAGE using 7.5% to 15% acrylamide linear gradient separating gels as described earlier (1).

C. RESULTS AND DISCUSSION

Data obtained from the fetuin-coated polystyrene tube binding inhibition experiments shown in Fig. II.1 confirm and extend previous observations (3,5). The graphs show that WGA and PT bind with variable avidity to five sialoglycoproteins containing similar sugar sequences. For example, fetuin, with

a 50% binding-inhibition concentration (IC_{50}) of 9.5 ± 1.6 x 10^{-8} M, was approximately 500 times better than the worst inhibitor, transferrin (IC_{50}, 4.3 ± 0.3 x 10^{-5} M) in the PT system (n = 2). Glycophorin A, with an IC_{50} of 4.5 ± 0.5 x 10^{-9} M, was at least 20,000 times better than the worst inhibitor, transferrin (IC_{50}, > 1.1 ± 0 x 10^{-4} M) in the WGA system (n = 2). This selectivity among ligands could permit PT to sample a subfraction of glycoprotein receptors from within a larger population bearing similar sugar sequences.

We next used affinity-isolation procedures to identify PT and WGA receptors in goose erythrocyte membranes. Goose erythrocytes were chosen because the PT-mediated goose hemagglutination reaction is an assay for PT-binding activity (16). We also examined the interaction of goose erythrocyte membrane proteins with fetuin-agarose to evaluate non-specific binding to an agarose-immobilized, nonlectin protein. Cholera toxin (CT)-agarose was also used because CT was previously shown to have a higher affinity for sialoglycolipids than for sialoglycoproteins (6).

Although the protein bands indicated by the solid dots beside lane 3 of Figure II.2B from the WGA column were very intense on the autoradiogram, they represented only a subfraction of the proteins present in the iodinated goose erythrocyte extract (Figure II.2B, far right lane). In contrast, the unmarked, lower-molecular-weight (less than 93,000 [93 kDa]) erythrocyte proteins detected in the WGA-agarose GlcNAc fractions were clearly the dominant species in the total erythrocyte extract and were also present in small amounts in the final wash fractions obtained from all four of the affinity columns (Figure II.2A). Therefore, the WGA-agarose column was able to selectively bind to a group of goose erythrocyte receptors that appeared to contain WGA-specific glycan units, because they were readily released from the column by GlcNAc. The 127 kDa protein (Figure II.2B, arrowhead) may represent a high affinity wheat germ agglutinin receptor which was not completely removed by

GlcNAc or a protein which bound to WGA-agarose by an alternate mechanism.

Only the lower-molecular-weight range proteins were released from PT-agarose by GlcNAc. In addition, most of the lower-molecular-weight proteins observed in Figure II.2B, lane 1, were observed in the GlcNAc and diethanolamine fractions obtained from the CT-agarose columns and, to a lesser extent, from the fetuin-agarose columns (Figure II.2B, lanes 5, 6, 7, and 8. However, all of the higher-molecular-weight goose erythrocyte proteins released from WGA-agarose in the presence of GlcNAc appeared in the PT-agarose-diethanolamine fractions. The failure of GlcNAc to release PT-bound erythrocyte receptors clearly reflects a major difference in the behavior of PT- and WGA-agarose. This finding was also consistent with our observation (3) that GlcNAc was unable to inhibit PT-mediated goose erythrocyte agglutination.

To further investigate the reason for the binding of the lower-molecular-weight group of proteins to PT-agarose, iodinated goose erythrocyte extracts were passed through an affinity column containing histone-agarose (Figure II.3). Cationic histones were chosen because we suspected that binding of the low-molecular-weight species may be the result of electrostatic interactions between negative charges on sialoglycoproteins and positive charges on WGA, PT, and CT (10,15,18). In addition, we increased the NaCl concentration in all of the buffer solutions used in the affinity isolation procedure to inhibit ionic interactions.

The lower-molecular-weight proteins observed in Figure II.2B were also clearly visible in the final wash and in diethanolamine fractions from the histone-agarose column (Figure II.3, lanes 5 and 6), but not in the fractions obtained from negatively charged (17) fetuin-agarose (Figure II.3, lanes 7, 8). Therefore, it appeared that we may have been correct in our assumption that ionic interactions were responsible for the binding of the lower-molecular-weight

proteins to PT-, WGA-, and CT-agarose. Although, in the presence of 1 M NaCl, some lower-molecular-weight species were detected in the diethanolamine fractions from PT-agarose (Figure II.3, lane 2), the 27 K and 74 K proteins observed in the histone-agarose diethanolamine fractions (Figure II.3, lane 5, arrowheads) and the PT-agarose diethanolamine fractions from the experiment described earlier (Figure II.2B, lane 2) were not among them.

Except for a small amount of a 120 kDa band in the histone-agarose fractions (Figure II.3, lanes 5 and 6), the high-molecular-weight species marked by the solid dots in Figure II.3 and corresponding to the high-molecular-weight species in Figure II.2B, lanes 2 and 3, were only observed in the diethanolamine fractions from the PT-agarose column. Therefore, with the exception of the 120 kDa protein*, it seemed unlikely that the high-molecular-weight protein species were binding to PT-agarose by simple electrostatic interactions.

Since we reported previously (4) that iodination reduced the receptor-binding activity of PT in goose erythrocytes and Chinese hamster ovary cells and it seemed unlikely that iodination would alter the capacity of PT for electrostatic interactions with negatively charged protein species, the Triton-solubilized goose erythrocytes were passed through iodinated PT-agarose. Polyclonal anti-PT immunoglobulin was used to determine the amount of immunoreactive PT remaining in the iodinated PT-agarose preparations. Although the anti-PT immunoglobulin G-binding experiment demonstrated that iodinated PT-agarose affinity columns retained at least 65% of the PT present in the untreated PT-agarose preparations, iodinated PT-agarose (Figure II.3, lanes 3 and 4) failed to bind any of the goose erythrocyte membrane proteins seen in Figure II.3, lane 2. This confirmed that the PT receptors

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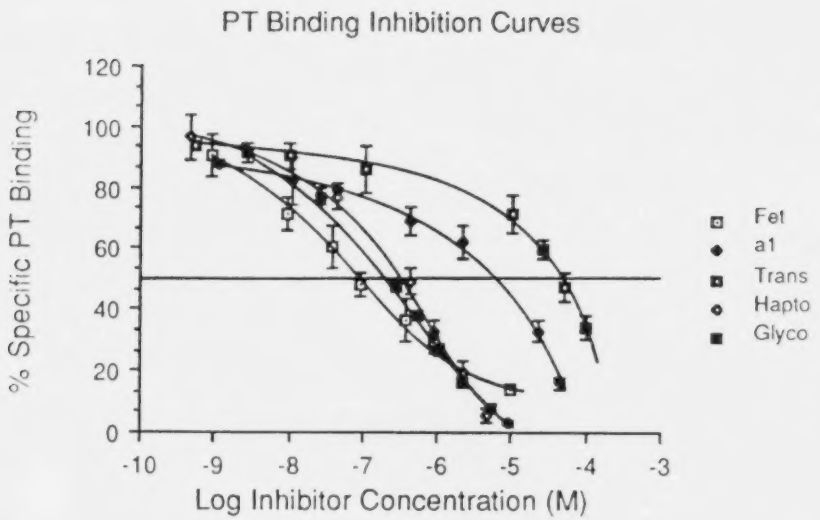
* Witvliet *et al.* (1989. Infect. Immun. **57**:3324-3330) found that PT dimers appeared to specifically bind to a 115 kDa erythrocyte protein that may be the same as our 120 kDa species.

were specific for iodination-sensitive PT-binding sites and were not binding by nonspecific electrostatic interactions.

The 92 kDa protein indicated by the arrowhead beside lane 3 in Figure II.3 was an enigma because, although it failed to bind to iodinated PT-agarose, it bound strongly to histone-agarose (Figure II.3, lane 6). It is possible that the 92 kDa protein contained sufficient negatively charged sialylated glycan units to bind to histone-agarose by electrostatic interactions, even in the presence of 1 M NaCl, and to PT-agarose by stereospecific interactions with the lectin-like binding sites.

Figure II.1

Inhibition curves ^{125}I-labeled PT (top) and ^{125}I-labeled WGA binding to fetuin-coated polystyrene tubes. The final concentration of ^{125}I-labeled PT or WGA in the assays was approximately 10^{-9} M. Background binding was determined in polystyrene tubes coated with bovine serum albumin alone, and the maximum (100%) binding in each experiment was measured in tubes containing no inhibitor. Binding at each concentration of inhibitor was performed in triplicate, and the error bars represent the standard error of the mean for each point. The glycoprotein inhibitors used in the experiments were fetuin (Fet), α1 acid glycoprotein (a1), transferrin (Trans), haptoglobin (Hapto), and glycophorin A (Glyco).


PT Binding Inhibition Curves
% Specific PT Binding
120
100
80
60
40
20
0
-10
-9
-8
-7
-6
-5
-4
-3
Log Inhibitor Concentration (M)
Fet
a1
Trans
Hapto
Glyco

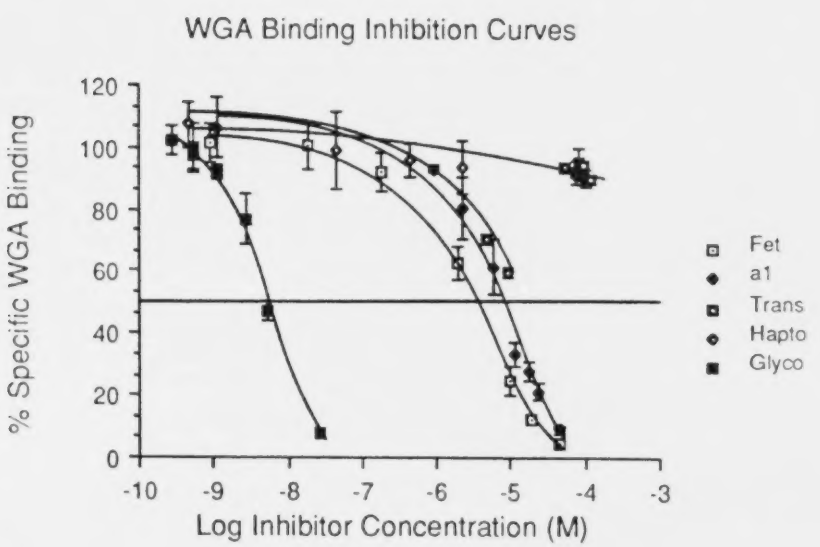

WGA Binding Inhibition Curves
% Specific WGA Binding
120
100
80
60
40
20
0
-10
-9
-8
-7
-6
-5
-4
-3
Log Inhibitor Concentration (M)
Fet
a1
Trans
Hapto
Glyco

Figure II.2.

SDS-PAGE analysis of goose erythrocyte proteins on affinity columns. (A) SDS-PAGE analysis of the final wash fractions obtained from PT-agarose (PT-Agar.), WGA-agarose (WGA-Agar.), CT-agarose (CT-Agar.), or fetuin-agarose (FET-Agar.) affinity columns. (B) SDS-PAGE analysis of bound, ^{125}I-labeled goose erythrocyte proteins released from the indicated columns by solutions containing 0.4 M GlcNAc or 50 mM diethanolamine (pH 11.5) containing 0.15 M NaCl. The PT-agarose and CT-agarose columns contained approximately 100 µg of protein, the WGA-agarose column contained approximately 165 µg of WGA, and the fetuin-agarose column contained approximately 230 µg of fetuin (based on a sialic acid content of 13.6 sialic acid residues per mol of fetuin [17]). The affinity chromatography samples were analyzed on 7.5% to 15% linear acrylamide separating gels in the presence of 2-mercaptoethanol as described earlier (1,8). The starting material that was loaded onto columns was analyzed in the far right lane. The positions of the protein standards are indicated by their molecular weights (10^{-3}) on the left side of the panels. The alpha spectrin band in human erythrocyte membranes was used for the 250 kDa molecular weight marker protein (9).

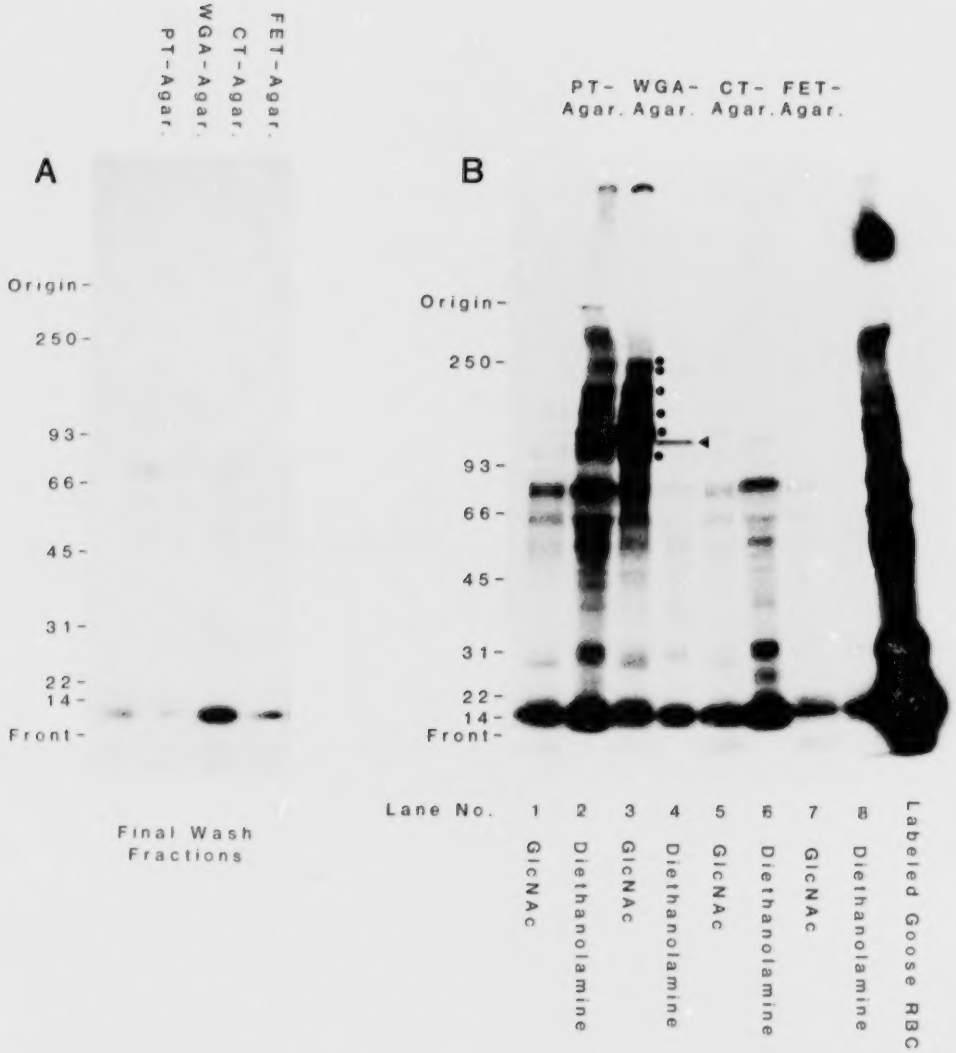
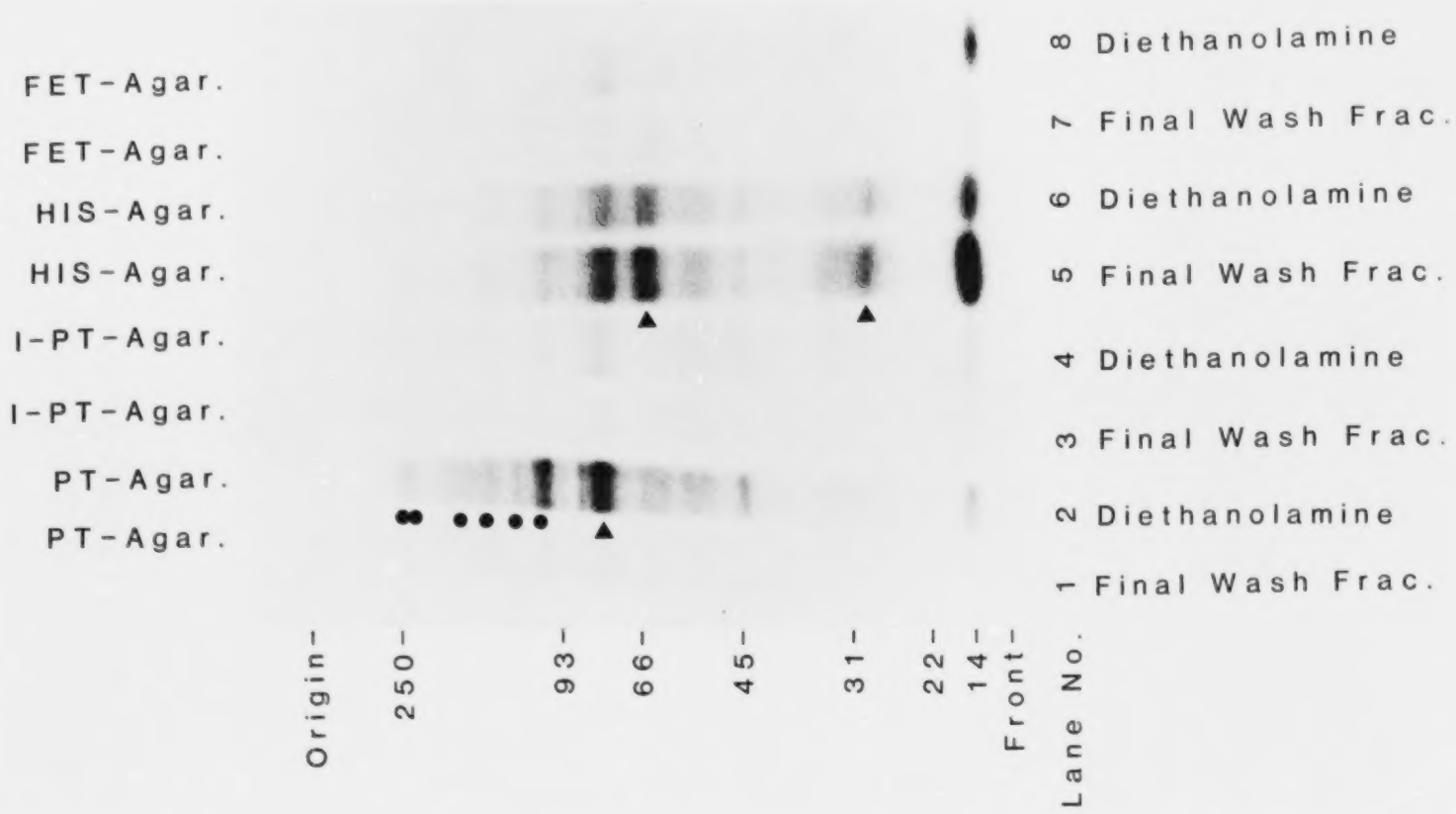

A
PT-Agar.
WGA-Agar.
CT-Agar.
FET-Agar.
Origin-
250-
93-
66-
45-
31-
22-
14-
Front-
Final Wash Fractions
B
PT- Agar.
WGA- Agar.
CT- Agar.
FET- Agar.
Origin-
250-
93-
66-
45-
31-
22-
14-
Front-
Lane No. 1 2 3 4 5 6 7 8
GlcNAc
Diethanolamine
GlcNAc
Diethanolamine
GlcNAc
Diethanolamine
GlcNAc
Diethanolamine
Labeled Goose RBC

Figure II.3.

SDS-PAGE analysis of goose erythrocyte proteins on affinity columns after washing with 1 M NaCl. SDS-PAGE analysis of ^{125}I-labeled goose erythrocyte proteins in the final wash fractions (Final Wash Frac.) or released from PT-agarose (PT-Agar.), iodinated-PT-agarose (I-PT-Agar.), histone-agarose (HIS-Agar.), or fetuin-agarose (FET-agar.) affinity columns by 50 mM diethanolamine. The conditions for the affinity-chromatography procedure were the same as those described previously (2), with the exception that the concentration of NaCl was increased to 1 M in all of the buffer solutions. The histone-agarose columns contained approximately 100 μg of protein. Iodinated PT-agarose was prepared by exposing a portion of the PT-agarose preparation to NaI (40 μM) in the presence of chloramine T (7 mM) for 30 min. The iodination procedure was repeated three times. Receptors were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis as described in the legend to Figure II.2.


FET-Agar.
FET-Agar.
HIS-Agar.
HIS-Agar.
I-PT-Agar.
I-PT-Agar.
PT-Agar.
PT-Agar.
Origin-
250-
93-
66-
45-
31-
22-
14-
Front-
Lane No.
1 Final Wash Frac.
2 Diethanolamine
3 Final Wash Frac.
4 Diethanolamine
5 Final Wash Frac.
6 Diethanolamine
7 Final Wash Frac.
8 Diethanolamine

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PREFACE TO CHAPTER III

The results of the affinity isolation experiments described in Chapter II convinced me that this procedure exhibited several of the potential drawbacks discussed in the preface to Chapter II. Because this process does not readily distinguish between high and low affinity binding interactions, it was difficult to define which of the higher molecular weight proteins interacting specifically with PT-agarose were important for PT binding to intact erythrocytes. In addition, the 120 kDa protein apparently bound to PT-agarose through electrostatic interactions even though conditions of high ionic strength were used. This protein was very similar to the 116 kDa PT-binding protein on goose erythrocytes found by Witvliet *et al.* (3) using immunoblot analysis, suggesting that the two proteins might be the same and that the immunoblot procedure might not distinguish specific from non-specific binding. I was also concerned that the requirement for surface labeling of plasma membrane proteins could lead to a failure to demonstrate receptors if they had no exposed tyrosines available for iodination.

To address these concerns, we adopted a photoaffinity labeling procedure to identify plasma membrane receptors for PT in intact cells. The advantages of this technique result from the unique properties of the photoaffinity crosslinking reagents, sulfosuccinimidyl 2-(*p*-azidosalicylamido)ethyl-1,3′-dithiopropionate (SASD; see figure III.2, below) and N-[4-(*p*-azidosalicylamido)butyl]-3′-(2′pyridyldithio)propionamide (APDP). SASD contains a terminal succinimidyl group that reacts with free amino groups at pH values greater than 7, resulting in release of the succinimidyl moiety and covalent insertion of the remainder of the crosslinker (*p*-azidosalicylamido)ethyl-1,3′-dithiopropionate; ASD) into the protein for which receptors are being investigated. The other end of the crosslinker terminates in a photoactivable phenyl azide that can also be labeled with ^{125}I. Activation of the

azido group with UV light causes insertion of this moiety into carbon-carbon and carbon-hydrogen bonds (1,2), resulting in the covalent linkage of the photoaffinity probe (eg. PT) to the receptor. Due to the presence of an internal disulfide bridge in the crosslinker, the resulting crosslinked complexes can be dissociated in the presence of sulfhydryl reducing reagents. This leaves the ^{125}I-labeled phenyl azido group attached to the receptor and facilitates its subsequent identification by SDS-PAGE. APDP is the only other crosslinker available commercially that has these unique properties, and differs in that it inserts into sulfhydryl rather than amino groups in proteins.

Though goose erythrocytes must express plasma membrane proteins responsible for PT-mediated hemagglutination, binding to these proteins is not known to produce intracellular changes or signalling events in these cells. In addition, we were not certain whether these proteins shared all the properties of PT receptors on other eukaryotic cell types (see Chapter I.C). T lymphocytes and Jurkat cells were therefore used to develop the photoaffinity labeling technique, since more is known about the effects of PT on these cells than any other cell type (see Chapter I.D) and because the effects of PT on T lymphocytes are important both in disease and in human responses to pertussis vaccine.

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CHAPTER III

LYMPHOCYTE RECEPTORS FOR PERTUSSIS TOXIN[1]

A. INTRODUCTION

Pertussis toxin (PT) is a major virulence factor produced by *Bordetella pertussis*, the etiological agent of whooping cough (14). PT is also a protective antigen and has adjuvant-like activity (15) in the killed, whole cell pertussis vaccine which is used to immunize children in North America (6). PT provides another example of an A-B class of bacterial toxin (25). It is composed of an enzymatic A (S1) subunit and a B oligomer which is required for toxin binding to receptors on host cell membranes, erythrocytes from several mammalian or avian species, and glycoproteins like fetuin (3,20,28,30). The B oligomer also interacts with as yet unidentified receptors on T lymphocytes and induces generalized lymphocyte proliferation (24,26) subsequent to the rapid intracellular accumulation of the second messengers calcium and diacylglycerol. Calcium is mobilized by PT from extracellular sources, presumably through the opening of membrane calcium channels (24). Apparently, this effect is not mediated through modification of intracellular GTP-binding proteins (G proteins) (13,18), though signal transduction through the CD3-T cell receptor complex may be involved (8,27). Both intact PT and the isolated B oligomer are only weakly mitogenic in the absence of compounds which directly activate protein kinase C *in vitro* or of accessory cells *in vivo* (23), suggesting that PT may act in concert with other stimulatory ligands to transmit the mitogenic signal.

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[1] A version of this chapter has been published. **Clark, C. G.** and G. D. Armstrong. 1990. Infect. Immun. **58**:3840-3846.

To obtain a better understanding of the molecular details of the PT B oligomer activity in T lymphocytes we have taken two approaches to identifying T lymphocyte surface proteins to which PT binds. The first approach utilized the affinity isolation technique to identify PT binding proteins in surface iodinated, detergent solubilized T lymphocytes. The second approach utilized an iodinatable, photoaffinity reagent, sulfosuccinimidyl 2-(*p*-azidosalicylamido)ethyl-1,3′-dithiopropionate (SASD), to pass radioactive label from modified PT to T lymphocyte receptor proteins in intact lymphocyte membranes. Our results suggested that PT binding to a 70,000-molecular-weight-protein in T lymphocyte membranes may represent the initial step in the process of PT mediated T lymphocyte activation.

B. MATERIALS AND METHODS

1. Preparation of lymphocytes.

Peripheral blood monocytic cells (PBMC) were prepared by centrifuging buffy coats obtained from fresh human blood into Ficoll-Paque (Pharmacia). The PBMC were then washed three times with sterile sodium phosphate buffered physiological saline (PBS, pH 7.2) and suspended in sterile PBS for the direct photoaffinity labeling experiments. Alternatively, to prepare T lymphocytes, the PBS-washed PBMC were suspended in FBS-supplemented RPMI 1640 at a concentration of approximately 1 x 10^8 cells/mL and 1 mL volumes were applied to nylon wool (Fenwal Laboratories) columns in FBS-supplemented RPMI 1640 as described earlier (9). After incubating the columns for 1 h at 37°C, non-adherent T lymphocytes were eluted with pre-warmed RPMI and maintained at 4°C until use. T cells were enumerated using anti-Leu-4 fluorescein isothiocyanate-conjugated antibodies (Becton Dickinson).

2. Surface iodination procedure.

Jurkat cells were grown in RPMI 1640 growth medium (Gibco) supplemented with 10% heat inactivated fetal bovine serum (FBS, Gibco). Before iodination, T lymphocytes or Jurkat cells were washed three times with PBS to remove serum proteins and suspended at a final concentration of 1×10^8 cells per mL in PBS. A 100 µL sample of the resulting cell suspensions was then incubated with 4 MBq Na[^{125}I] (629 GBq/mg, Edmonton Radiopharmaceuticals) in the presence of 1,3,4,6-tetrachloro-3α,6α-diphenylglycouril (IODO-GEN; Pierce) for 1 minute. The iodinated cells were washed three times with PBS to remove unbound radioactive iodine and solubilized in Triton X-100 as reported previously (2). Next, the Triton-solubilized cells were added to PT-agarose, histone-agarose, or fetuin-agarose affinity columns and incubated for 15 minutes at room temperature. The procedure for preparing the agarose derivatives is described in our earlier report (29 [Chapter II]). Unbound proteins were removed by washing the columns with 5 mL of 50 mM sodium phosphate buffer, pH 7.4, containing 1 M NaCl (wash buffer), and the final 0.5 mL wash fraction was collected for analysis. Bound proteins were then eluted with 100 µL of 50 mM diethanolamine (pH 11.5) containing 0.15 M NaCl (7), followed by 400 µL wash buffer. The fractions were dialyzed overnight at 4°C against 0.1% SDS in distilled water and lyophilized using a Savant model SVC100H Speed Vac concentrator. The dried samples were dissolved in 50 µL of Laemmli sample buffer (10) containing 10% (vol/vol) 2-mercaptoethanol and analyzed by SDS polyacrylamide gel electrophoresis (PAGE) using 7.5% to 15.0% linear acrylamide separating gels (2,10). The gels were dried under vacuum using a Bio Rad model 1125B slab gel drier and exposed at -80°C to Kodak X-Omat AR X-ray film using Du Pont Cronex Lightning Plus intensifying screens.

3. ^{125}I labeling of SASD and preparation of ^{125}I-ASD-PT.

^{125}I-2-(*p*-azidosalicylamido)ethyl-1,3´dithiopropionate (ASD)-PT was prepared by the method of Shephard *et al.* (21), with the following changes. All reactions were carried out in the dark or under dim lighting conditions in aluminum foil-wrapped disposable glass culture tubes (12 by 75 mm). SASD (Pierce) was dissolved in 0.33 M sodium phosphate (pH 7.5) containing 5% (vol/vol) dimethylsulfoxide. The iodination reactions were carried out in IODO-GEN-coated culture tubes (12 by 75) containing 10 µg SASD in 100 µL buffer and 20 MBq Na[^{125}I] (629 GBq/mg). The reactions were allowed to proceed for 1 minute and the ^{125}I-SASD was removed from the IODO-GEN tube and incubated for 30 min with 10 µg of PT bound to 50 µL (bed volume) of fetuin-agarose (4). Next, the fetuin-agarose suspension was transferred to a glass wool-plugged Pasteur pipet and washed with 10 to 15 mL PBS. ^{125}I-ASD-PT was eluted from the fetuin-agarose using 200 µL of 50 mM diethanolamine (pH 11.5) containing 0.15 M NaCl (7). The ^{125}I-ASD-PT was collected in a culture tube containing 200 µL of 100 mM Tris-HCl (pH 4.4) and the pH of the resulting solution was adjusted to approximately 6.5 by using 1 N HCl. The amount of ^{125}I-ASD-PT recovered from the fetuin-agarose was determined using the trypsinized goose erythrocyte agglutination assay (4) and the protein dye binding assay described by Bradford (5). The specific activity of the preparations was determined by TCA precipitation (4). Radioactivity was quantified in an LKB model 1270 Rackgamma II gamma counter.

4. Photoaffinity labeling of lymphocytes.

Cells for the photoaffinity labeling experiments were first washed four times with 15 mL volumes of PBS to remove unbound serum components. Approximately 0.1 µg (2.5×10^5 cpm) of freshly prepared ^{125}I-ASD-PT was added to 100 µL of PBS containing 3×10^7 T lymphocytes, 1×10^6 Jurkat cells, or 5×10^6 PBMC. Approximately 30 times more T lymphocytes were

used in the experiments to compensate for the size difference between them and Jurkat cells. The ^{125}I-ASD-PT was allowed to react with the cells in the presence or absence of 1000 fold excess underivatized PT in the dark for 90 minutes on ice. The mixtures were then exposed to a UV light source (emission maximum, 302 nm) for 10 minutes at a fixed distance of 11 cm. Next, the cell suspensions were washed twice with 2.5 mL PBS, dissolved in SDS sample buffer containing 10% (vol/vol) 2-mercaptoethanol and analyzed by SDS-PAGE using 12.5% acrylamide separating gels. In reactions receiving no cells, an equivalent amount of ^{125}I-ASD-PT in PBS was exposed to UV light and applied to the gels.

5. Immunoblotting procedure.

Jurkat cell proteins were transferred from SDS polyacrylamide gels to nitrocellulose membrane (Bio-Rad) in a Bio-Rad Transblot apparatus for 16 h at 27 V (constant). The transfer buffer was 25 mM sodium phosphate, pH 7.5. The nitrocellulose membranes were then incubated for 1 hour at room temperature with 10 mL of 0.05% Tween 20 in PBS (blocking buffer). The PBS-Tween buffer was removed and rabbit anti-bovine serum albumin (BSA) or anti-human serum albumin (HSA) antibodies dissolved in fresh blocking buffer were added to duplicate blots. The blots were incubated for an additional 2 h at room temperature. After washing, bound antibodies were detected using horseradish peroxidase-conjugated second antibody (Sigma).

C. RESULTS

1. Identification of PT receptors in T lymphocytes by affinity isolation.

The results of experiments designed to identify PT receptors in T lymphocytes by affinity chromatography are displayed in Figure III.1. In these experiments at least 6 well resolved bands, migrating in the 45,000 to 250,000

molecular weight range, were found to bind to PT-agarose but not to agarose containing covalently bound histone or fetuin. Therefore, these six bands are potential lymphocyte receptors for PT. As discussed in our previous article (29), the diffuse material observed in the diethanolamine fractions obtained from both PT and histone, but not fetuin-agarose, probably represents anionic membrane components interacting non-specifically with polycationic proteins such as histone or the B oligomer of PT but not interacting with anionic proteins such as fetuin (25).

2. Identification of Jurkat cell receptors by using ^{125}I-ASD-PT.

The next set of experiments used the ^{125}I-labeled photoaffinity labeling reagent, SASD, to investigate PT receptors in the Jurkat T lymphocyte cell line. SASD has been used to identify lipopolysaccharide-specific binding proteins in lymphoid cells (11,12,31). The technique is most useful for investigating receptors for large or aggregated ligands because, as indicated in the scheme presented in Figure III.2, the resulting crosslinked complexes can be dissociated in the presence of sulfhydryl reducing reagents. This leaves the ^{125}I-labeled probe attached to the receptor and facilitates its subsequent identification by SDS-PAGE.

When fetuin-agarose bound PT was reacted with ^{125}I-SASD the amount of protein recovered in the diethanolamine fractions ranged from 23% to 40% of the total PT (10 µg) applied. The average amount of ^{125}I-ASD-labeled PT obtained in 0.4 mL was 4.0 ± 3.3 µg (n = 8) as determined by the Bradford dye binding assay (5) or 2.3 ± 1.5 µg (n = 8) as determined by agglutination of trypsinized goose erythrocytes (4). The difference between the amount of PT recovered as determined by the two assays was not significant ($p > 0.2$) and therefore it was apparent that all of the PT recovered from fetuin-agarose retained its ability to agglutinate goose erythrocytes. Otherwise, the goose hemagglutination assay

would have indicated that significantly less PT was recovered in the diethanolamine fractions.

Incorporation of radioactive label into TCA precipitable ^{125}I-ASD-PT averaged 84.4 ± 9.2% (n = 8) in the absence of 2-mercaptoethanol and 9.5 ± 6.9% in the presence of sulfhydryl reducing reagent. This observation was consistent with the incorporation of approximately 90% of the ^{125}I into the ASD portion of the derivatized PT. SDS-PAGE analysis of ^{125}I-ASD-PT in the absence of reducing reagent (Figure III.3) indicated that all of the PT subunits were labeled, though ^{125}I-ASD was incorporated preferentially into subunit S2. The low amount of label incorporated into the S1 subunit reflects the absence of lysine groups in this molecule. The mean specific activity of the eight ^{125}I-ASD-PT preparations was 3.3 ± 1.3 x 10^6 cpm/μg of PT and represented an average efficiency of labeling of roughly 15% (range 9% to 42%), assuming one molecule of radioactive iodine per ASD.

Noncovalently attached PT bound tightly enough to the surface of Jurkat cells that it was not removed by washing with PBS. Therefore, it was not possible to determine how much of the noncovalently bound ^{125}I-ASD-PT was converted to covalently bound ^{125}I-ASD-PT by exposure to UV light because neither of them could be removed from the cells using non-destructive methods.

When ^{125}I-ASD-PT labeled Jurkat cells were analyzed by SDS-PAGE in the presence of 2-mercaptoethanol (Figure III.4B, lane 1) a protein band was observed which had a calculated M_r of 70,000 ± 2,400 [n = 4]. This protein was not detected when ^{125}I-ASD-PT was irradiated with UV light in the absence of Jurkat cells (Figure III.4B, lane 3), nor was it detected in unirradiated ^{125}I-ASD-PT-Jurkat cell complexes (data not shown). Only lower-molecular-weight proteins were detected in the absence of Jurkat cells. These co-migrated with the five PT subunits (subunits S4 and S5 were not resolved in this SDS-PAGE system) observed in the Coomassie stained gel (Figure III.4A, lane 2) and probably represent the products

of endogenous subunit-subunit labeling or subunits that were directly iodinated because of residual Na[^{125}I] in the original ^{125}I-SASD preparation.

The amount of ^{125}I-ASD that was actually incorporated into the 70,000-Da protein after UV irradiation of ^{125}I-ASD-PT-labeled Jurkat cells was less than 1% of the total label added to the reaction mixtures. This was determined by counting the radioactivity in the 70,000-Da receptor bands that were cut from the dried gels. Nonetheless, when the procedure was carried out in the presence of 1000-fold-excess unmodified PT (Figure III.4B, lane 2) the amount of label transferred to the 70,000-Da receptor was reduced by 75%, demonstrating that unmodified PT was able to compete for receptor labeling with ^{125}I-ASD-modified PT and suggesting that the two ligands (ASD-PT and unmodified PT) were binding to the same receptor species. The reduction of incorporation of label into receptor was determined by comparing the radioactivity in receptor bands which were cut from dried gels and by scanning the autoradiograms in a densitometer. The two procedures gave the same results.

3. Identification of T lymphocyte receptors for PT by photoaffinity labeling.

A 70,000-Da band was also observed when human peripheral blood T lymphocytes (about 92% T lymphocytes) were probed with ^{125}I-ASD-PT in the absence of unmodified PT (Figure III.5A, lane 1). In addition, although some labeling of the PT subunits was seen, the 70,000-molecular-weight T lymphocyte band was greatly reduced (77%) in the presence of unmodified PT (Figure III.5A, lane 3) or (as in the Jurkat system discussed earlier) in T lymphocytes that were not exposed to UV light prior to analysis by SDS-PAGE (Figure III.5A, lane 2). Again, this observation is consistent with the photoinduced formation of covalent linkages between ^{125}I-ASD-PT and a 70,000-molecular-weight receptor in T lymphocytes.

It concerned us that the ^{125}I-ASD-PT-labeled lymphocyte band had approximately the same relative mobility on SDS gels as fetuin and HSA or BSA. To address the possibility that the 70,000-molecular-weight protein may be a contaminant derived from serum we probed immunoblots to determine if the PBS-washed Jurkat cells contained either BSA or HSA. Neither BSA nor HSA were detected in Jurkat cells that were probed with anti-BSA or anti-HSA (Figure III.6). Moreover, the 70,000 dalton band was also apparent in human ^{125}I-ASD-PT-labeled PBMC (approximately 70% of which represented T lymphocytes) which had not been exposed to FBS during their isolation (Figure III.5B). Therefore, it is also unlikely that the 70,000 Dalton protein detected by photoaffinity labeling was fetuin which would have been present in FBS used in the preparation of both Jurkat cells and T lymphocytes.

D.DISCUSSION

Although all of the lymphocyte components observed in the molecular weight range from 45,000 to 250,000 in Figure III.1 (lane 2) appeared to bind specifically to PT-agarose the results of the affinity isolation experiments must be interpreted with caution. For example, the assumption that all T-lymphocyte proteins capable of binding to PT are efficiently labeled with iodine may not be correct and, consequently, important receptor components that may not be iodinated could easily be overlooked. The procedure also suffers from the necessity of having to solubilize the iodinated lymphocyte membranes with detergent prior to performing affinity chromatography. This may expose cryptic receptors which might never bind to PT in the native state. Alternatively, it is possible that important receptors may fail to bind to PT in the presence of detergent.

Many of these potential problems may be avoided by using the photoaffinity labeling technique. With photoaffinity probes it is possible to investigate receptors in intact

membranes. This may prevent inappropriate (cryptic) receptors from being identified and avoids the potential problem of loss of receptor activity in the presence of detergents. Moreover, the lack of chemical specificity demonstrated by the azido group of the photoprobe (22) insures maximum reactivity with receptors to which the modified ligand is bound. Therefore, the procedure also circumvents the requirement for specific chemical groups such as tyrosine residues in the case of surface iodination or free amino groups in the case of aminoreactive bifunctional crosslinking reagents.

Despite the many advantages, photoaffinity labeling procedures are not without their own unique problems. Since chemical groups capable of forming covalent bonds with the receptor must be introduced into the ligand, it becomes necessary to demonstrate that the chemically modified ligand still recognizes the same receptors as the unmodified ligand. This is usually accomplished by demonstrating that the amount of affinity-labeled product is reduced when most of the available receptor binding sites are occupied by the unmodified ligand (1,17,19).

Specific activity calculations indicated that the incorporation of ^{125}I-SASD into PT ranged from a low of 0.09 ^{125}I-ASD to a high of 0.42 ^{125}I-ASD molecules per toxin molecule. The average incorporation (0.15 ^{125}I-ASD/PT) was about 3 times lower than that reported for ^{125}I-ASD-LPS preparations (31). Nonetheless, although the efficiency of PT labeling was low, underivatized PT was always able to compete for transfer of ^{125}I-ASD to the 70,000-molecular-weight receptor protein in Jurkat cells, T lymphocytes, and PBMC. The apparent difference between experiments in the efficiency of the photoaffinity labeling reaction are a reflection of the amount of ^{125}I-SASD incorporated into PT and hence the amount of ^{125}I-ASD that was available for transfer to the 70,000-molecular-weight receptor. This finding clearly demonstrates that both ^{125}I-ASD-PT and underivatized PT bind

to the same receptors on the surfaces of T-lymphocyte and Jurkat cells.

When ^{125}I-ASD-PT was used, only one PT receptor was identified in T lymphocytes that was not observed when the reaction was performed in the presence of unmodified PT. Nonetheless, it is possible that the 70,000 molecular weight protein may not be directly involved in delivering the mitogenic signal to the cell. The 70,000-molecular-weight receptor may, in fact, be only one part of a more complex multisubunit receptor system. Other signal transduction components may not have been labeled because, for one reason or another, they may not have been oriented correctly for the attachment of the ^{125}I-ASD probe. Some of these receptor subunits might be represented by the additional proteins detected by the affinity isolation procedure (Figure III.1). The observation that one of the protein bands that bound specifically to PT-agarose in the affinity isolation experiment had a molecular weight of approximately 70,000 (arrow, Figure III.1) is consistent with the idea of an oligomeric PT receptor complex containing a 70,000-molecular-weight subunit, identified by affinity chromatography and photoaffinity labeling, and additional subunits that were only detected by affinity isolation. The inability, because of geometrical constraints, to identify all subunits in an oligomeric PT receptor system represents a potential limitation of the photoaffinity labeling procedure. The use of photoaffinity labeling reagents with variable spacing between the functional endgroups could be useful in resolving this issue.

It was interesting that ^{125}I-SASD was incorporated preferentially into lysine groups of the S2 subunit of PT (Figure III.3) despite the fact that a computer program for predicting secondary structure of proteins indicated that the same number of lysine residues should be exposed on the surfaces of subunits S2, S3 and, S4. Previously, Nogimori *et al.* (16) presented data suggesting that lysine groups in PT

dimer 2 (composed of subunits S3 and S4) were important for PT's ability to bind to receptors on mouse lymphocytes. More recent evidence (30) suggests that dimer 1 (composed of subunits S2 and S4) is more important than dimer 2 for PT binding to sialoglycoproteins such as fetuin and goose erythrocyte receptors. According to the data of Nogimori et al., the goose hemagglutination activity of PT was not altered by modification of lysine groups in dimer 1. Our data are consistent with those of Nogimori *et al.* because SASD modification of lysines in subunit S2 did not appear to affect the ability of ^{125}I-ASD-PT to agglutinate goose erythrocytes. However, it was apparent from the data in Figure III.3 that free amino groups in subunit S2 were extensively modified by ^{125}I-SASD despite the fact that the coupling reaction was performed while PT was bound to fetuin-agarose. This suggests that lysines on subunit S2 that were labeled by ^{125}I-SASD are not located close to the fetuin or goose erythrocyte receptor binding site in dimer 1 because, if they were, fetuin should have prevented them from becoming labeled. In light of the data of Nogimori *et al.* (16) suggesting that lysine groups in subunits S3 and S4 may be important for the interaction of PT with lymphocyte receptors, it is possible that the relative resistance of lysines in subunits S3 and S4 to modification by the SASD reagent was responsible for the sparing of the ability of ^{125}I-ASD-PT to bind to receptors in T lymphocytes.

Figure III.1

SDS-PAGE analysis of PT receptors obtained from human T lymphocytes by affinity chromatography. The sample preparation, SDS-PAGE, and autoradiography techniques are described in Materials and Methods. Lanes: 1, 3, and 5, material derived from the final 0.5 mL wash fraction from each of the affinity columns; 2, 4, and 6, proteins from the diethanolamine fractions. The arrow indicates a protein with a relative mobility similar to the 70,000 Dalton species detected by the photoaffinity labeling technique. The positions to which the protein standards migrated are indicated by their molecular weights (in thousands) on the left.

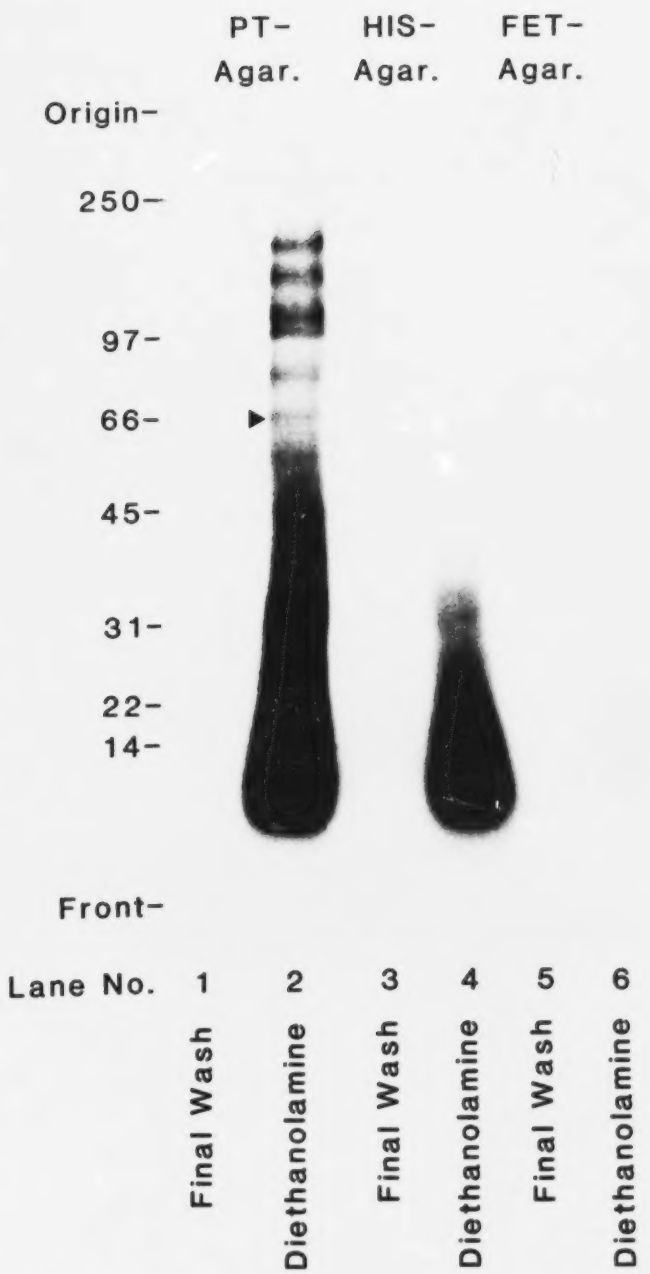
PT-
Agar.
HIS-
Agar.
FET-
Agar.
Origin-
250-
97-
66-
45-
31-
22-
14-
Front-
Lane No. 1 2 3 4 5 6
Final Wash
Diethanolamine
Final Wash
Diethanolamine
Final Wash
Diethanolamine

Figure III.2

Schematic illustration of the photoaffinity labeling technique. Step 1. Covalent attachment of ^{125}I-SASD to free amino groups of PT at alkaline pH. Step 2. Non-covalent interaction of ^{125}I-ASD-PT with receptors in the dark. Step 3. Covalent attachment of the photo-activated azido group of ^{125}I-ASD-PT to the PT receptor. Steps 4 and 5. Reduction of the disulfide bond of ^{125}I-ASD-PT and resolution of PT from the labeled receptor during SDS-PAGE analysis.

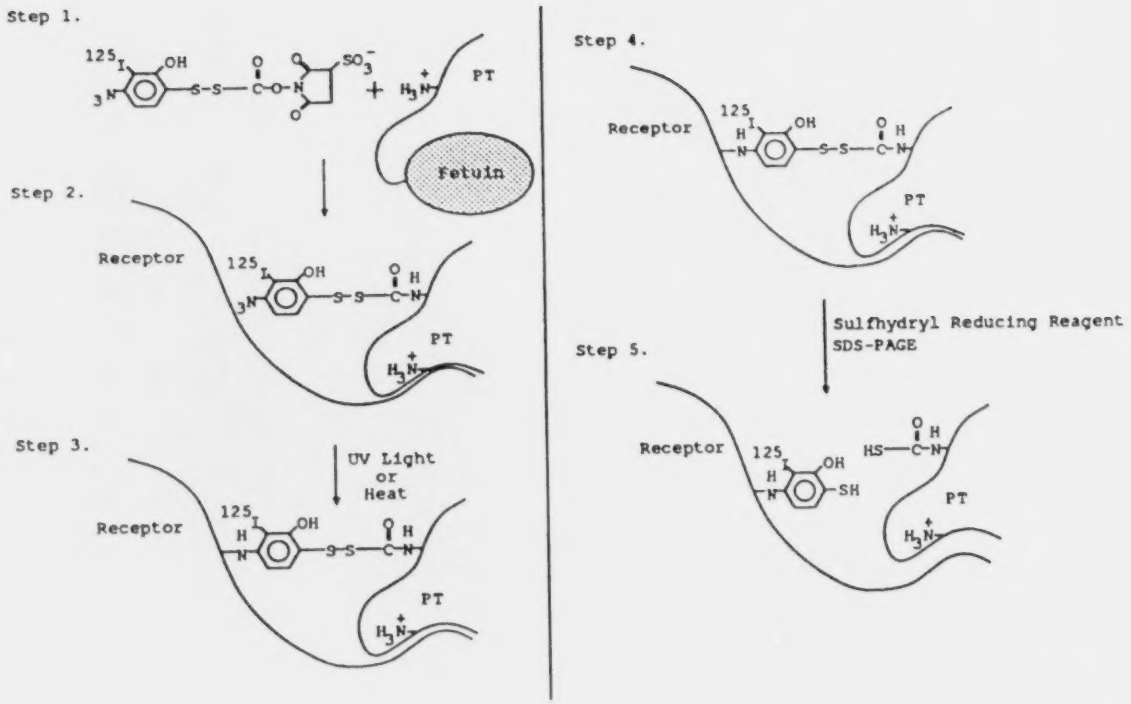
Step 1.
PT
Fetuin
Step 2.
Receptor
PT
Step 3.
UV Light
or
Heat
Receptor
PT
Step 4.
Receptor
PT
Sulfhydryl Reducing Reagent
SDS-PAGE
Step 5.
Receptor
PT

Figure III.3

PT subunits labeled with ^{125}I-ASD. ^{125}I-ASD-PT was analyzed by SDS-PAGE in the absence of sulfhydryl reducing reagent using a 16% separating gel and prepared for autoradiography as described in Experimental Procedures. PT subunits derivatized with ^{125}I-ASD migrated to positions shown by arrows in the figure. The positions to which the protein standards migrated are indicated by their molecular weights (x 1000) to the left of the photograph.

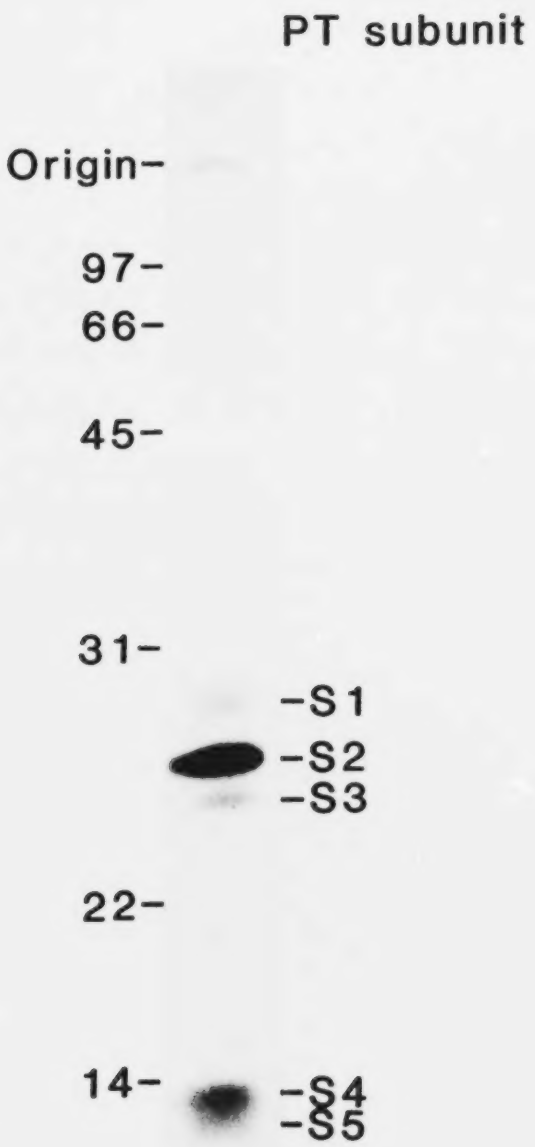
PT subunit
Origin-
97-
66-
45-
31-
-S1
-S2
-S3
22-
14-
-S4
-S5

Figure III.4

SDS-PAGE analysis of photoaffinity labeled Jurkat cells. Photoaffinity labeled Jurkat cells were analyzed by SDS-PAGE in the presence of 2-mercaptoethanol using 10% separating gels. The Coomassie stained gel is shown on the left and the corresponding autoradiogram is shown on the right. ^{125}I-ASD-PT was used to photoaffinity label Jurkat cells in the absence (autoradiogram, lane 1) and presence (autoradiogram, lane 2) of excess unmodified PT. An equivalent amount of ^{125}I-ASD-PT to that in lane 1 but exposed to UV light in the absence of Jurkat cells was applied to lane 3 of the gel. Proteins comigrating with the PT subunits are indicated by the symbols.

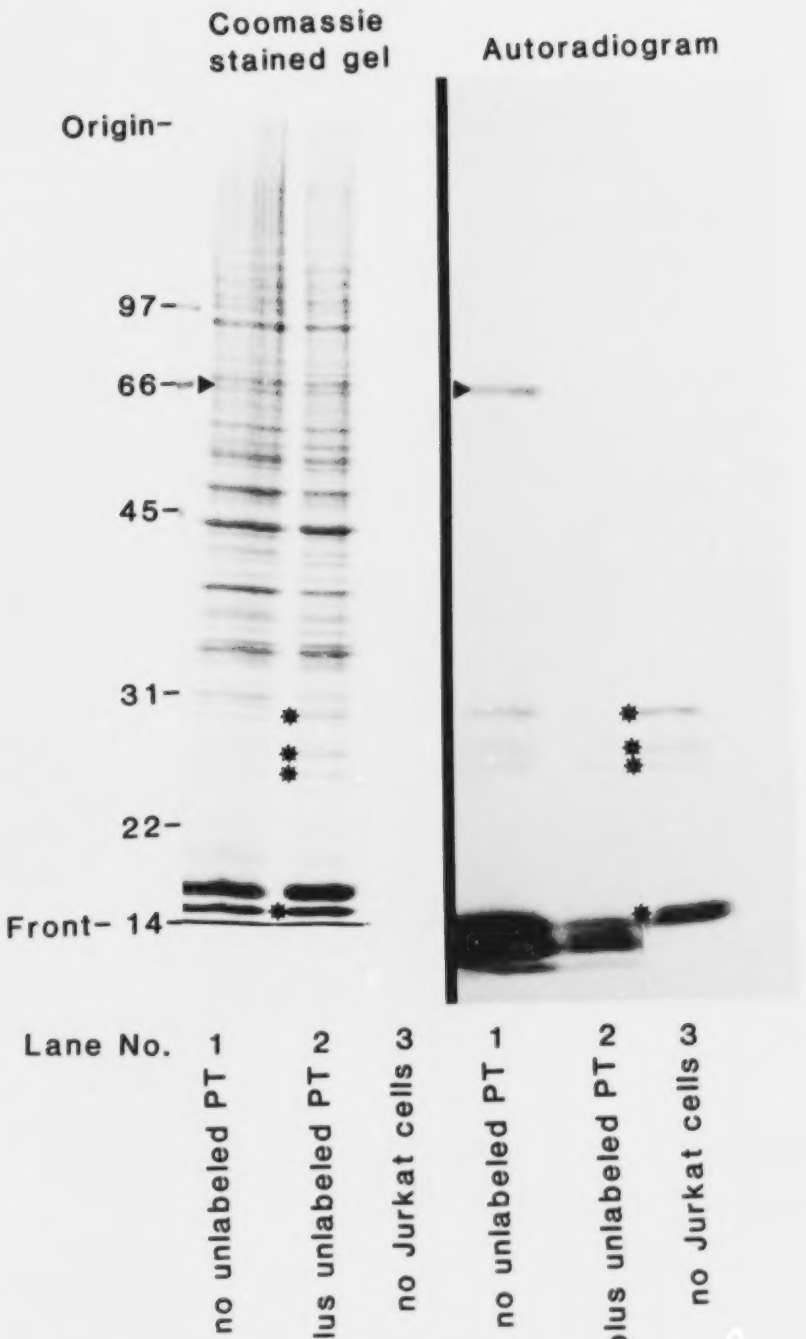
Coomassie stained gel
Autoradiogram
Origin-
97-
66-
45-
31-
22-
Front- 14
Lane No. 1
no unlabeled PT
2
plus unlabeled PT
3
no Jurkat cells
1
no unlabeled PT
2
plus unlabeled PT
3
no Jurkat cells

Figure III.5

SDS-PAGE analysis of photoaffinity labeled T lymphocytes and PBMC. Panel A. T lymphocytes were affinity labeled with ^{125}I-ASD-PT in the absence (lane 1) or presence (lane 2) of a 1000 fold molar excess unmodified PT. Autoradiogram of 7.5% to 15% linear acrylamide separating gel. Panel B. PBMC were affinity labeled with ^{125}I-ASD-PT. Autoradiogram of a 10% acrylamide separating gel.

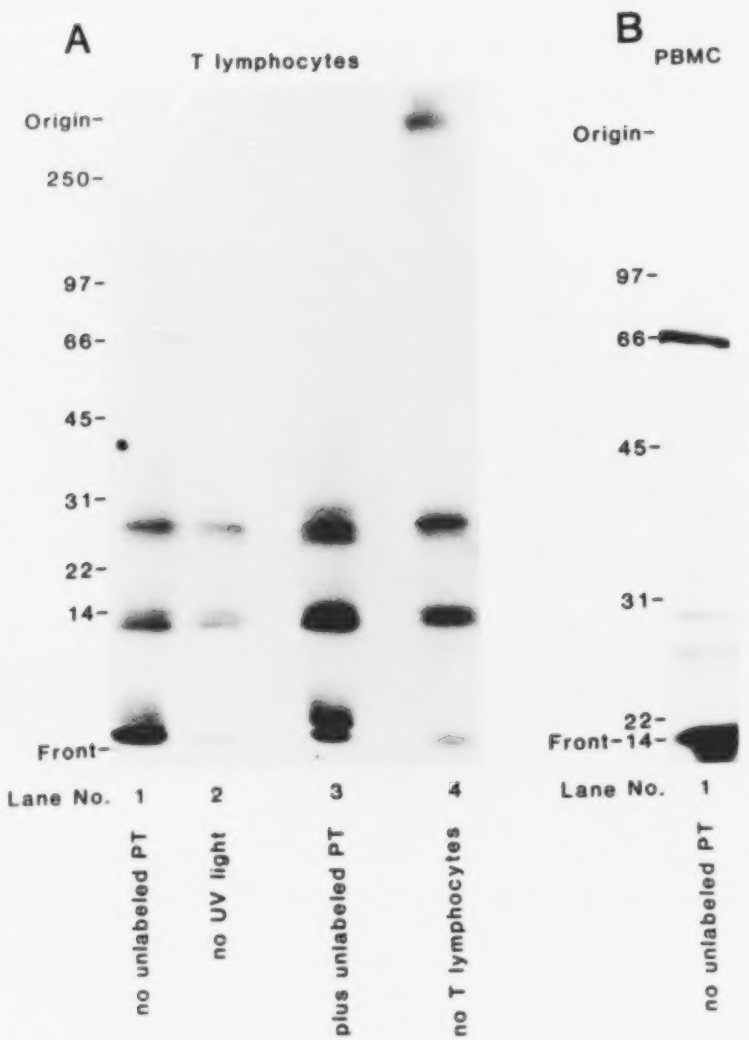
A
T lymphocytes
Origin-
250-
97-
66-
45-
31-
22-
14-
Front-
Lane No. 1 2 3 4
no unlabeled PT
no UV light
plus unlabeled PT
no T lymphocytes
B
PBMC
Origin-
97-
66-
45-
31-
22-
14-
Front-
Lane No. 1
no unlabeled PT

Figure III.6

Western blot analysis of Jurkat cells using anti-BSA and anti-HSA probes. Solubilized Jurkat cell proteins from 5 x 10^5 Jurkat cells, purified HSA, and protein standard containing BSA were analyzed by SDS-PAGE using 12.5% separating gels, electrophoretically transferred to nitrocellulose, and probed with anti-BSA or anti-HSA as described in the Experimental Procedures.

plus anti-HSA
plus anti-BSA
Origin-
BSA
Front-
Lane No.
1
2
1
2
Jurkat cells
HSA
Jurkat cells
protein standard

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PREFACE TO CHAPTER IV

During the time the research reported in chapter III was being completed, a new photoaffinity crosslinking agent became available. This agent, N-[4-(*p*-azidosalicylamido)-butyl]-3´-(2´pyridyldithio)propionamide (APDP), covalently modifies sulfhydryl groups rather than free amino groups in protein ligands. We felt that the use of this agent would provide an important control for the previous experiments. It would not modify potentially important lysine residues in dimer 2 (S3; see Chapter I.C), and may bind with a different spatial distribution to the surface of the PT holotoxin.

At the same time, we became aware of the work of Morrison and his colleagues, who have identified LPS receptors on lymphoid cells from several different species (5,6). The similarities in the apparent molecular weights of these LPS receptors and the Jurkat cell PT-receptor identified by our research were striking. Though the apparent molecular weight of the dominant LPS receptor was originally calculated to be approximately 80 kDa (3-6), subsequent investigations led to the calculation of a refined molecular weight of 73 kDa for this protein (2). Furthermore, the ^{125}I-ASD-LPS-labeled receptor was found at exactly the same position as BSA in one-dimensional SDS-PAGE gels (2), as was the ^{125}I-ASD-PT receptor we identified. Finally, the LPS receptor was found to be a glycoprotein (1) with an isoelectric point of approximately 6.5 (5). LPS is known to activate B lymphocytes and macrophages, and it may also have subtle effects on T lymphocytes (6). These results are supported by the finding that serum-dependent binding of LPS (2) to the dominant 73 kDa glycoprotein receptor was found on B and T lymphocytes from various species (5). We therefore investigated the similarities between receptors for PT and LPS in Jurkat cells in more detail.

Though the oligosaccharide structures important for high affinity binding of PT to glycoproteins have been examined in

detail (see Chapter I.C.1), nothing was known about the specificity of PT binding to cellular receptors. Sialic acid-specific lectins and glycoproteins were therefore used to determine whether oligosaccharides are involved in PT interactions with Jurkat cells and identify the oligosaccharide structures involved.

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CHAPTER IV

Characterization of the pertussis toxin receptor on T lymphocytes and Jurkat cells[1]

A. INTRODUCTION

Pertussis toxin (PT) is an important virulence factor produced by *Bordetella pertussis*, the etiological agent of whooping cough (31), and is an immunoprotective component of whole cell and acellular vaccines against this disease (5,12,24,34). PT has also been extensively used as a tool for the identification of GTP-binding proteins (G proteins) involved in different intracellular signalling pathways of various cell types (35,36). From these kinds of studies and from investigations into the role of PT in the disease process, it has become apparent that PT can interact with and modify the function of many different host cells and tissues. It is therefore important to characterize completely the biological effects of PT in order to understand the importance of PT in producing disease.

PT is an oligomeric A-B type toxin that contains an enzymatically active A subunit which is responsible for catalyzing the ADP-ribosylation of a specific cysteine residue in the carboxy-termini of G_α proteins (19), uncoupling these proteins from the normal regulatory pathways and thus producing many of the biological effects of the toxin (14). PT's pentameric B oligomer is responsible for toxin binding to receptors on host cell membranes, lymphocytes, other cell types such as goose erythrocytes and Chinese hamster ovary (CHO) cells, and to several sialylated glycoproteins (1,42,47-49,51). Similar lectin-like

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[1] A version of this chapter has been submitted for publication. Portions of the work were presented at the 1991 Annual Meeting of the Western Branch of the Canadian Society of Microbiologists. Winner of the best poster award.

interactions may also contribute to the attachment of *Bordetella pertussis* to epithelial cells lining the upper respiratory tract of humans (49).

Once bound to the cell surface, the A subunit of PT passes through the plasma membrane and modifies its intracellular targets (4). The activity of the A protomer is not always required for PT to produce biological effects in target cells. Binding of the B oligomer to T lymphocyte cell surface receptors is sufficient to produce intracellular signals capable of stimulating a mitogenic response (47,48). Since the ability of PT to bind to cells will determine which cells are subsequently affected, and since binding may itself produce biological effects, it is important to identify and characterize receptors for PT in order to fully understand its activity.

We have previously used the photoaffinity crosslinking technique to specifically label a 70 kDa PT-binding protein on both human peripheral T lymphocytes and on Jurkat cells (7). A protein having a similar apparent molecular weight in SDS-PAGE gels was one of two PT-binding proteins identified by Rogers *et al.* (39) on peripheral blood lymphocytes and Jurkat cells. More recently, a 70 kDa protein was found to be the dominant binding site for bacterial cell wall peptidoglycan on mouse B and T lymphocytes and macrophages (9). This protein was found to be identical to the 73 kDa LPS-binding protein on mammalian lymphocytes and macrophages previously identified by Morrison and his colleagues (10,27,29,38).

These data suggested that the same or, at least a very similar, protein may bind PT, LPS, and peptidoglycan, as well as other mitogenic ligands. To explore this hypothesis we identified PT-binding proteins on Jurkat cells using the photoaffinity crosslinkers, sulfosuccinimidyl-2-(*p*-azido-salicylamido)ethyl-1,3′-dithiopropionate (SASD) and N-[4-(*p*-azido-salicylamido)butyl]-3′-(2′pyridyldithio)propionamide (APDP). We also investigated Jurkat cell receptors for LPS

and two plant lectins previously shown to have similar oligosaccharide binding specificities as PT (17) and determined the pI of the photoaffinity labeled PT receptor in T lymphocytes and Jurkat cells.

B. MATERIALS AND METHODS

1. Materials. The following reagents were obtained from the Sigma Chemical Co. (St. Louis, MO): laminin, fibrinogen, transferrin, Trizma base, Trizma hydrochloride, glycine, and diethanolamine. SASD, APDP, and 1,3,4,6-tetrachloro-3α,6α-diphenyl-glycoluril (IODO-GEN) were from the Pierce Chemical Co. (Rockford, IL). Acrylamide, bis-acrylamide, N,N,N′,N′-tetramethylethylenediamine (TEMED), molecular weight standards for SDS-PAGE, and Coomassie blue stain were from Bio-Rad (Mississauga, Ont.). *Sambucus nigra* agglutinin (SNA) and *Maackia amurensis* leukoagglutinin (MAL) were from Boehringer Mannheim Canada (Laval, Que.). *Salmonella minnesota* wild type LPS was from List Biologicals Inc. (Campbell, CA). PT was a generous gift from the Connaught Centre for Biotechnology Research (Willowdale, Ont.). Ampholytes used in the isoelectric focusing procedure were Pharmalyte pH 3-10 or LKB 3.5-10 ampholytes from Pharmacia LKB Biotechnology (Uppsala, Sweden). Chloramine T and X-Omat AR X-ray film were from Kodak Canada (Vancouver, B.C.). T lymphocytes and Jurkat cells were obtained as described earlier (7), and were grown in RPMI 1640 medium containing 10% fetal bovine serum (FBS) without antibiotics.

2. Preparation of ^{125}I-ASD-PT, -SNA, -MAL, and ^{125}I-APD-PT. ^{125}I-ASD- and ^{125}I-APD-conjugates were prepared using the protocol described previously (7; Chapter III). All reactions were carried out under reduced lighting in foil-wrapped 12 x 75 mm borosilicate culture tubes. Ten µg of SASD or APDP in 100 µl of sodium phosphate-buffered physiological saline (PBS, pH 7.2) was iodinated with 3.7 MBq of Na[^{125}I]

for 1 min in tubes coated with 40 µg of IODO-GEN. The mixture was then removed from the IODO-GEN tube and immediately added to 1.5 ml Eppendorf tubes containing approximately 10 to 15 µg of PT or lectin bound to 100 µl (bed volume) of fetuin-agarose. PT was not treated with reducing agents prior to incubation with the ^{125}I-APDP. After incubation for 30 min at ambient temperature, the agarose gel suspensions were transferred to glass wool-plugged Pasteur pipets, washed with approximately 15 ml of PBS, and eluted with 200 µl of 50 mM diethanolamine (pH 11.5) containing 0.15 M NaCl (6). The pH of the resulting solutions was immediately adjusted to approximately 7 with 1 N HCl. The amount of ^{125}I-ASD-derivatized PT or lectin recovered was determined using the chymotrypsin-treated goose erythrocyte agglutination assay described previously (2). The specific activity of the preparations was determined by trichloroacetic acid precipitation (2) in the presence and absence of reducing agents (7; Chapter III).

3. Preparation of ^{125}I-ASD-LPS. The results of Lei *et al.* (26) indicated that *Salmonella minnesota* wild type LPS bound to the same receptor as the *E. coli* 0111:B4 LPS. ^{125}I-ASD-LPS was therefore prepared by the method of Wollenweber and Morrison (52) using *Salmonella minnesota* wild type LPS. All procedures were performed under reduced lighting using foil-wrapped containers. Sonicated LPS (1 mg in 500 µl of water) was added to 0.4 mg of SASD. Borate buffer (500 µl; 0.1 M, pH 8.5) was then added, and the resulting mixture was incubated at room temperature for 30 min. The LPS mixture was again sonicated, 0.4 mg of fresh SASD was added, and the solution was incubated for an additional 30 min. The ASD-LPS was centrifuged at 2000 x g for 5 min, dialyzed against PBS overnight at 4°C, and, when necessary, stored at -80° C. Approximately 0.1 mg (100 µl) of ASD-LPS was iodinated for 10 min in the presence of 18.5 MBq Na[^{125}I], 10 µl of 0.1 mg/ml chloramine T (dissolved in water), and 10 µl of 0.1 mM KI

(dissolved in water). The iodination reaction was terminated by adding 30 µl of 0.1 mg/ml $Na_2S_2O_3$ (dissolved in water), and the ^{125}I-ASD-LPS was added to a 10 ml volume (0.8 x 19.5 cm) Sephadex G-50 column that had been pre-equilibrated with 0.2% gelatin in PBS. The column was eluted with PBS, and 0.5 ml fractions were counted in an LKB model 1270 Rackgamma II gamma counter to identify fractions containing ^{125}I-ASD-LPS. These fractions were pooled, dialyzed overnight against PBS, and stored at 4° C until use.

4. Photoaffinity labeling of cells. Photoaffinity labeling was performed as described previously (7; Chapter III). Cells were first washed four times with 15 ml volumes of PBS to remove media components, especially serum albumin. Approximately 0.1 to 0.4 µg of ^{125}I-ASD- or ^{125}I-APD-derivatized PT or lectins was added to approximately 1 x 10^6 Jurkat cells or 1 x 10^7 human T lymphocytes. The cell suspensions were then incubated in the dark for 90 min at 4°C with occasional shaking. Competitors of the photoaffinity crosslinking were added 15 min before the ^{125}I-ASD- or ^{125}I-APD-conjugates. After incubation, the cell suspensions were exposed to UV light (254 nm emission maximum, 11 cm distance) for 10 min, then washed twice with 2.5 ml of PBS. The crosslink-labeled cells were immediately used for one-dimensional SDS-PAGE or two-dimensional SDS-PAGE. One-dimensional SDS-PAGE samples were dissolved in SDS sample buffer containing 50 mM dithiothreitol and analyzed using 12.5% acrylamide separating gels. After SDS-PAGE, the gels were dried under vacuum in a Bio-Rad model 1125B slab gel dryer and exposed at -80° C to X-ray film using Du Pont Cronex Lightning-Plus intensifying screens.

5. Two-dimensional SDS-PAGE. Two dimensional SDS-PAGE was performed according to the method of O'Farrell (33), as described by Dunbar (8). Separation in the first dimension was accomplished using isoelectric focusing on 1 x 130 mm

tube gels with 2% Pharmalyte (pH 3-10) or 2% LKB ampholyte (pH 3.5-10). After isoelectric focusing, the tube gels were loaded onto 12.5% SDS-PAGE gels and the proteins were separated in the second dimension. Duplicate isoelectric focussing tube gels were cut into 5 mm pieces and incubated for 2 h in 2 ml of degassed water obtained from a Milli Q water treatment system. The pH gradient obtained during the isoelectric focussing step was determined by measuring the pH of the resulting solutions (33). Two-dimensional gels were stained with 0.125% Coomassie blue R250 in 50% methanol/10% acetic acid (v/v), destained with 10% methanol/10% acetic acid (v/v), and subjected to autoradiography as described above.

C. RESULTS

1. Photoaffinity labeling of Jurkat cells using ^{125}I-ASD-modified lectins.

^{125}I-ASD photoaffinity derivatives of SNA and MAL were prepared to investigate their receptors on Jurkat cells. The ^{125}I-ASD-derivatives of both lectins retained their ability to agglutinate chymotrypsin-treated goose erythrocytes, indicating that their binding properties were not affected by the addition of ^{125}I-ASD groups. SDS-PAGE analysis of Jurkat cells incubated with ^{125}I-ASD-SNA or ^{125}I-ASD-MAL revealed that the two lectins photoaffinity labeled a protein with the same apparent molecular weight (70 kDa) as the protein labeled by ^{125}I-ASD-PT (Figure IV.1a, lane 1; Figure IV.1b, lanes 1 and 4). Labeled proteins with molecular weights of 33 kDa (Figure IV.1b, lanes 1, 3 and 7) represented autolabeled SNA, while the two bands with molecular weights of about 32 and 36 kDa (Figure 1b, lanes 4, 6, and 8) represented autolabeled MAL. Only these bands (arrows) were observed when Jurkat cells were omitted from the reaction mixtures (Figure IV.1b, lanes 7 and 8). The arrows beside lane 1 in Figure

IV.1a indicate autolabeled PT subunits as discussed in chapter III (7).

As predicted, the 70 kDa protein was not labeled by either ^{125}I-ASD-SNA or by ^{125}I-ASD-MAL in the absence of UV light (Figure IV.1b, lanes 3 and 6, respectively). Further, the inclusion of excess unmodified SNA or MAL caused a decrease in the incorporation of label into the 70 kDa protein (Figure IV.1b, lanes 2 and 5 respectively) when ^{125}I-ASD-SNA or ^{125}I-ASD-MAL were used in the photoaffinity labeling reactions. The ability of the unmodified lectins to compete for binding and photoaffinity labeling by the modified lectins showed that both modified and unmodified lectins bound to the same receptor.

Densitometric analysis of the autoradiograms (for example, Figure IV.1a) from inhibition studies using the two lectins revealed that labeling of the 70 kDa protein by ^{125}I-ASD-PT was reduced by 83% ± 24 (n = 2) by a thousand fold excess of underivatized MAL and by 59% ± 3 (n = 2) by a similar excess of underivatized SNA. Underivatized PT reduced the labeling of the 70 kDa receptor by ^{125}I-ASD-SNA or ^{125}I-ASD-MAL by 95% (Figure 1c, lanes 2 and 4). These results suggested that PT, SNA and MAL recognized the same or similar 70 kDa receptor proteins on Jurkat cells, albeit with different affinities. The protein with an apparent molecular mass of approximately 50 kDa to 56 kDa in Figure IV.1, panels a and c, was not labeled consistently (compare Figure IV.1b), but may correspond to the 50 kDa protein labeled by ^{125}I-APD-PT (Figure IV.3).

2. Effect of glycoproteins on labeling of Jurkat cells with ^{125}I-ASD-PT.

Sialic acid-containing glycoproteins having known binding affinities for PT (17,18) were used to compete for the ^{125}I-ASD-PT photoaffinity labeling of the 70 kDa Jurkat cell protein according to the protocol in Materials and Methods. When present in a hundred fold molar excess,

fibrinogen and laminin reduced the amount of radioactive label incorporated into the 70 kDa protein by 58% and 65%, respectively, while transferrin had no effect (Table IV.1). Higher concentrations of fibrinogen further inhibited the photoaffinity labeling in a dose-dependent manner. At five hundred and thousand fold molar excesses, transferrin was also capable of partially inhibiting photoaffinity labeling. This is consistent with transferrin's poor ability to inhibit PT-binding in ELISA inhibition assays (17). The photoaffinity labeling of the 70 kDa protein by ^{125}I-ASD-SNA and ^{125}I-ASD-MAL was inhibited by the glycoproteins in a manner consistent with the specificities of these lectins. Estimates of the inhibition of binding of the photoaffinity probes, made by comparing the densities of autolabeled PT, SNA, and MAL protein bands in the presence and absence of inhibitors, correlated very well with the values obtained for the inhibition of labeling (Table IV.1). These results further indicated that the PT receptor on Jurkat cells included sialylated oligosaccharide sequences capable of binding PT, SNA and MAL.

3. Identification of Jurkat cell receptors using ^{125}I-APD-PT.

A second crosslinking reagent (APDP) was used to prepare a photoaffinity PT derivative for further characterization of the PT-Jurkat cell interaction. APDP is covalently linked to proteins through interactions with free sulfhydryl groups, whereas SASD links through free amino groups. Since different chemical groups in PT are modified by APDP and SASD, we expected that each crosslinker would have a different spatial distribution on the surface of the PT molecule. We reasoned that this might put the crosslinkers in physical proximity to different Jurkat cell membrane proteins, resulting in a different labeling pattern.

^{125}I-APD-PT retained its ability to hemagglutinate goose erythrocytes, indicating that the binding activity of the PT

was unaffected by the addition of the APD group. In the absence of underivatized PT at least three proteins were photoaffinity crosslink-labeled in experiments using ^{125}I-APD-PT (Figure IV.2). One of these appeared to be the same as the 70 kDa protein labeled by ^{125}I-ASD-PT. A second protein with an apparent molecular weight of approximately 32 kDa (Figure IV.2) was labeled to a much greater degree by ^{125}I-APD-PT than the 70 kDa receptor, while a third protein with a molecular mass of 50 kDa was labeled at about the same intensity as the 70 kDa receptor. Though the 70 kDa and 50 kDa proteins were labeled to a greater or lesser degree in different experiments, they were always labeled to a much lesser extent than the 32 kDa protein when ^{125}I-APD-PT was used (see also Figures IV.3 and IV.4). Labeled proteins migrating below the 31 kDa molecular weight standard in Figure IV.2 represented autolabeled PT subunits. Photoaffinity labeling of the 32, 50, and 70 kDa bands by ^{125}I-APD-PT was reduced in the presence of fibrinogen and laminin, though not transferrin (Figure IV.3), as well as by SNA and MAL (Figure IV.4).

4. Comparison of Jurkat cell and T lymphocyte proteins photoaffinity labeled by ^{125}I-ASD-LPS and ^{125}I-ASD-PT.

We have shown previously that ^{125}I-ASD-PT photoaffinity labeled a 70 kDa protein on both peripheral blood T lymphocytes and on Jurkat cells (7; Chapter III). Analysis by two-dimensional SDS-PAGE demonstrated that the 70 kDa protein on T lymphocytes (Figure IV.5a) or Jurkat cells (Figure IV.5b) had a pI of 6.4 ± 0.2 (n = 4). The lymphocyte receptor for LPS and peptidoglycan identified previously (10,26) had a similar size and isoelectric point, suggesting that it might be the same protein as the 70 kDa PT and lectin receptor described in this article. To test this hypothesis we performed a series of experiments using ^{125}I-SASD-derivatized LPS from *Salmonella minnesota*.

^{125}I-ASD-LPS specifically labeled a 70 kDa Jurkat cell membrane protein that appeared to be the same as the protein

labeled by ^{125}I-ASD-PT (Figure IV.6a, lane 1) Two proteins with molecular masses of approximately 53 and 32 kDa were also labeled by ^{125}I-ASD-LPS. Excess unmodified LPS reduced the amount of label incorporated into these proteins (Figure IV.6a, lane 2). These results confirmed that the ^{125}I-ASD-derivative of *Salmonella minnesota* wild type LPS, which contains the O-antigenic oligosaccharide chain, photoaffinity labeled a protein with a similar molecular weight to the protein labeled by ^{125}I-ASD-derivative made with *E. coli* LPS. The presence of excess unmodified LPS did not reduce the amount of label incorporated into the 70 kDa protein when ^{125}I-ASD-PT was used for crosslinking Jurkat cells, while an excess of unmodified PT did compete for labeling (Figure IV.6b, lanes 2 and 3). The results of several experiments indicated that LPS neither enhanced or inhibited the photoaffinity labeling of Jurkat cell proteins by ^{125}I-ASD-PT.

D. DISCUSSION

PT, SNA and MAL all appear to specifically bind to a protein on Jurkat cells and T lymphocytes that has a molecular mass of 70 kDa and a pI of 6.4 ± 0.2. LPS photoaffinity labels a protein with very similar characteristics, suggesting that it may bind to the same receptor as PT, SNA and MAL. Peptidoglycan, PT, and MAL have previously been shown to be mitogenic for human T lymphocytes (11,22,41), although LPS was not (11). Dziarski recently demonstrated that similar sugar structures were functionally involved in the binding of LPS and peptidoglycan to a 70 kDa receptor in lymphocytes (9,10), though Lei *et al.* found that peptidoglycan did not bind to the 73 kDa LPS receptor on murine splenocytes (27). Since PT, SNA and MAL do not contain the disaccharide structures common to LPS and peptidoglycan, it is unlikely that they would interact with the same sites on a common lymphocyte receptor protein that bind peptidoglycan and LPS. The finding that excess unmodified LPS

failed to inhibit the ^{125}I-ASD-PT crosslinking of this protein (Figure IV.6b, lane 3) is consistent with ``this hypothesis. Alternately, LPS may interact with a separate glycoprotein having a very similar molecular mass and isoelectric point.

PT also specifically labeled Jurkat cell proteins with molecular masses of approximately 32 and 50 kDa when APDP was used as the crosslinking reagent (Figure IV.2), suggesting that these proteins may interact with the 70 kDa protein to form a receptor complex in the membranes of Jurkat cells. ^{125}I-ASD-LPS also photoaffinity labeled 32 and 53 kDa Jurkat cell proteins (Figure IV.6a, lane 1), further indicating the similarity of the receptors for PT and LPS. The length of APDP (21.02 Å) is very close to the length of SASD (18.9 Å), suggesting that differences in PT-labeled proteins arising from the use of the two different crosslinking agents were not due to the interaction of the crosslinker with proteins at varying distances from the bound PT. Differences in receptor labeling by the ^{125}I-ASD-PT and ^{125}I-APD-PT derivatives may have arisen because of differences in the spatial orientation of APDP and SASD on the surface of PT. This could also provide an explanation for the reduced autolabeling of ^{125}I-ADP-derivatized PT subunits (compare Figures IV.1a and IV.2). Alternatively, the differences in labeling may have arisen due to the insertion of the two crosslinking agents into different chemical groups in PT. Free amino groups in PT's dimer 2 (S3-S4) are thought to play a role in the binding of toxin to lymphocytes (32). While SASD could react with these groups, interfering with the binding of dimer 2 to cell-surface proteins, APDP would not. It is clear from these results that a single crosslinking agent may not label all of the receptor proteins interacting with a ligand, in this case PT, and that different crosslinking agents modifying dissimilar chemical groups in the ligand should be used whenever possible for photoaffinity labeling studies.

SNA and MAL are lectins that bind to glycoconjugates having terminal sialic acid residues attached to galactose by α(2-6) and α(2-3) linkages, respectively (43,50). These lectins photoaffinity labeled the same proteins as PT, competed for PT photoaffinity labeling of Jurkat cell proteins, and were competed in the photoaffinity labeling reaction by PT, indicating that a component of the PT, SNA, and MAL receptor contained oligosaccharide sequences having both α(2-3) and α(2-6) linked sialic acid residues. These results are consistent with the previously determined properties of PT, which is known to bind to glycoproteins attached to solid supports (17,51) and on cells (3,51). In addition, these results support our earlier findings (7; Chapter III) indicating that the PT receptor on lymphocytes is likely not a serum protein such as BSA or HSA, which do not contain *N*-linked oligosaccharides. Significantly, we have not been able to demonstrate PT binding to HSA in ELISA binding assays (see Chapter VI).

While the inhibition of ^{125}I-ASD-PT photoaffinity labeling by the lectins is not complete even when thousand fold excesses of the lectins were used, the reciprocal inhibition of lectin photoaffinity labeling by underivatized PT was complete at a five hundred fold excess of PT. This suggests that PT may bind to the Jurkat cell receptor with a relatively higher affinity than the two lectins. Alternately, this phenomenon may result from the tendency of PT to form aggregates (30), leading to a greater avidity of binding for aggregated PT compared to the non-aggregated lectins.

The inhibition of PT binding to, and photoaffinity labeling of, Jurkat cells by both laminin, which contains only α(2-3) linked sialic acid structures, and transferrin and fibrinogen, which contain exclusively α(2-6) linked sialic acid structures, supports these conclusions. We have used these glycoproteins to examine the lectin-like binding of PT (17) because, unlike fetuin, each has *N*-linked

oligosaccharides with sialic acid attached exclusively through one kind of linkage. The fact that both glycoproteins can inhibit the ^{125}I-ASD-PT mediated photoaffinity labeling of Jurkat cell receptors indicates that PT can recognize both the α(2-3) and α(2-6) sialic acid linkages, supporting and extending results obtained earlier using ELISA PT-binding inhibition assays (17) and with SNA and MAL (above). PT therefore appears to recognize similar oligosaccharide determinants on whole cells and isolated glycoproteins.

A 43 kDa protein similar to the one labeled by Rogers *et al.* (39) did not appear in autoradiograms after labeling cells with either ^{125}I-APD-PT or ^{125}I-ASD-PT, even though a similar photoaffinity labeling procedure was used. It is possible that this 43 kDa protein may represent a fourth protein in an, as yet hypothetical, PT receptor complex. These authors demonstrated that a 66 to 70 kDa protein similar to the one shown here was labeled by ^{125}I-ASD-PT. They concluded, however, that the interaction resulting in the labeling of this protein was non-specific based on the fact that the labeling of this protein could not be inhibited using a hundred fold excess of underivatized PT. We feel that the ability of underivatized PT to completely inhibit this photoaffinity labeling adequately demonstrates the specificity of the reaction. The requirement for large amounts of underivatized PT to achieve complete inhibition of photoaffinity labeling may reflect a higher concentration of cell surface receptors for PT than is usually seen in high affinity, low capacity pharmacological systems. Alternatively, aggregation of PT may result in the interaction of several toxin molecules with each receptor, leaving sufficient numbers of receptors available to bind both ^{125}I-ASD-PT and unmodified PT at lower competitor concentrations.

PT binding to T lymphocytes, B lymphocytes, neutrophils, pancreatic islet cells, and Jurkat cells causes an influx of calcium from extracellular sources (20,21,23,28,40,44) which

occurs in conjunction with other intracellular signalling events and changes in cellular metabolism (15). The calcium influx stimulated by PT in Jurkat cells is a result of the early production of inositol phosphates, with an initial release of calcium from intracellular stores (45). In human peripheral blood T lymphocytes, however, PT does not appear to affect the turnover of inositol phosphates, but may mediate signalling through other, uncharacterized pathways with minimal involvement of intracellular calcium sources (46,47).

These findings suggest that PT may directly interact with membrane calcium channel complexes in human T lymphocytes (46). Several surface antigens present on T lymphocytes, including CD2, CD3, CD4, CD5, CD6, CD7, CD8, and Tp44, are known to cause calcium mobilization after crosslinking with antibodies (25). The PT receptors we have identified do not appear to be CD2, CD3, CD6, CD7, or CD8, since these antigens have different molecular weights in SDS-PAGE gels under reducing conditions. Preliminary results indicate that the 70 kDa protein is not CD5, since this 70 kDa protein is not precipitated using antiserum specific for this antigen (unpublished findings) and since anti-CD5 antibodies do not compete for photoaffinity labeling of this protein by ^{125}I-ASD-PT, ^{125}I-ASD-SNA, or ^{125}I-ASD-MAL (Figure IV.7).

The relationship of CD4 to the 70 kDa protein is currently under investigation, although reports that PT can deliver mitogenic signals to both $CD4^+$ and $CD8^+$ T lymphocytes (16) indicate that the 70 kDa receptor may not be the CD4 antigen. Robinson *et al.* (37) have recently shown that the glycophosphatidyl inositol (GPI) anchor present on many T lymphocyte proteins is directly involved in the delivery of activation signals to the cell, possibly through inositol triphosphate-stimulated opening of membrane calcium channels (13). Further work is required to determine which activation

pathways are coupled to the PT receptor in Jurkat cells and T lymphocytes.

Table IV.1

Inhibition of photoaffinity labeling of the 70 kDa Jurkat cell protein by ^{125}I-ASD-PT, -SNA, and -MAL using glycoproteins.

	molar excess	% inhibition of labeling[1] ^{125}I-ASD-PT	^{125}I-ASD-SNA	^{125}I-ASD-MAL
fibrinogen	100x	58	53	4
	500x	48	78	ND[2]
	1000x	82	100	30
laminin	50x	56	ND	68
	100x	65	22	ND
transferrin	100x	0	10	0
	500x	31	45	ND
	1000x	63	63	13

[1] Glycoproteins were used to compete for the ^{125}I-ASD-PT photoaffinity labeling of the 70 kDa Jurkat cell protein according to the protocol in Materials and Methods. The inhibition of photoaffinity labeling was calculated from densitometer scans of autoradiograms by comparing the density of the 70 kDa protein band in the absence and presence of inhibitors.

[2] ND means not determined.

Figure IV.1

SDS-PAGE analysis of Jurkat cells photoaffinity labeled with ^{125}I-ASD-PT (panel a), ^{125}I-ASD-SNA or ^{125}I-ASD-MAL (panels b and c). Jurkat cells were photoaffinity labeled with the three ^{125}I-ASD derivatives as described in the Materials and Methods section in the presence or absence of underivatized lectins as indicated at the bottom of the figure. After photoaffinity labeling, the PBS-washed Jurkat cells were dissolved in SDS-PAGE sample buffer containing 50 mM dithiothreitol (DTT) and analyzed on 12.5% polyacrylamide separating gels. The gels were then stained, dried and exposed to X-ray film to produce the autoradiograms presented in the figure. The positions to which the protein standards migrated are indicated on the left of each panel by a number representing their molecular weights (x 10^{-3}). The dye front and interface between the stacking and separating gels (origin) are also indicated in the figure. Arrowheads refer to the autolabeled PT subunits (panel a), SNA, and MAL subunits (panel b).

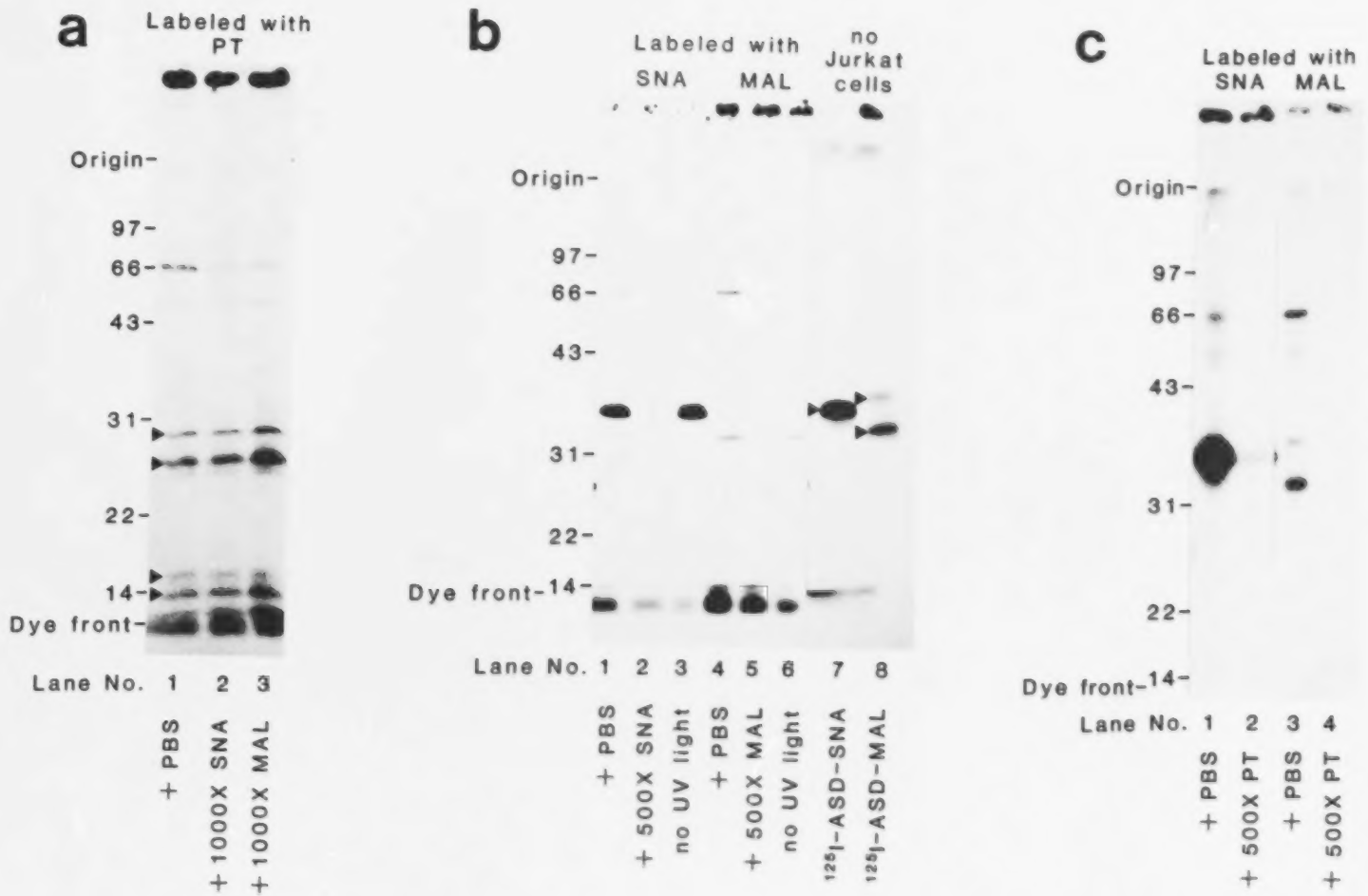

a
Labeled with PT
Origin-
97-
66-
43-
31-
22-
14-
Dye front-
Lane No. 1 2 3
+ PBS
+ 1000X SNA
+ 1000X MAL
b
Labeled with SNA
MAL
no Jurkat cells
Origin-
97-
66-
43-
31-
22-
14-
Dye front-
Lane No. 1 2 3 4 5 6 7 8
+ PBS
+ 500X SNA
no UV light
+ PBS
+ 500X MAL
no UV light
125I-ASD-SNA
125I-ASD-MAL
c
Labeled with SNA MAL
Origin-
97-
66-
43-
31-
22-
14-
Dye front-
Lane No. 1 2 3 4
+ PBS
+ 500X PT
+ PBS
+ 500X PT

Figure IV.2

SDS-PAGE analysis of Jurkat cells photoaffinity labeled with ^{125}I-APD-PT. Jurkat cells were photoaffinity labeled with ^{125}I-APD-PT as described in the Materials and Methods section in the presence or absence of underivatized PT as indicated at the bottom of the figure. The autoradiogram used to produce the figure was prepared as described in the legend to Figure 1. The positions to which the protein standards migrated are indicated (molecular weights x 10^{-3}) on the left of the panel.

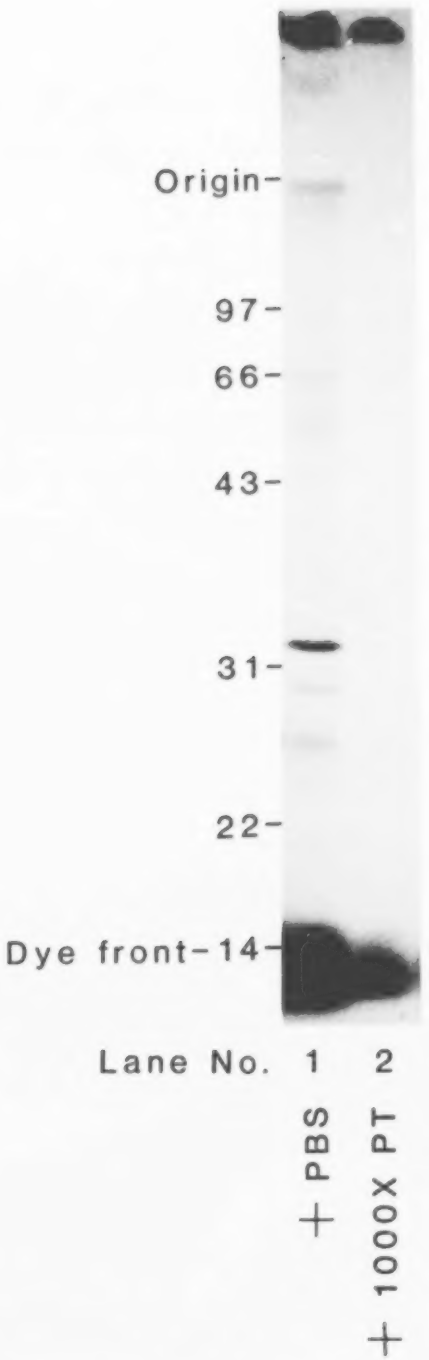

Origin-
97-
66-
43-
31-
22-
Dye front-14-
Lane No. 1 2
+ PBS
+ 1000X PT

Figure IV.3

Inhibition by glycoproteins of photoaffinity labeling of Jurkat cells using ^{125}I-APD-PT. Jurkat cells were photoaffinity labeled in the presence of the glycoproteins shown in the figure by the procedure outlined in Materials and Methods, then were analyzed by SDS-PAGE and autoradiography as described in the legend to Figure IV.1. The positions to which the protein standards migrated are indicated on the left of each panel by a number representing their molecular weights (x 10^{-3}). The dye front and interface between the stacking and separating gels (origin) are also indicated in the figure. The 67 to 70 kDa protein is labeled with an arrowhead, the 32 kDa protein with a filled circle, and autolabeled PT subunits are labeled with stars. The information in this figure was included as "data not shown" in the submitted manuscript.

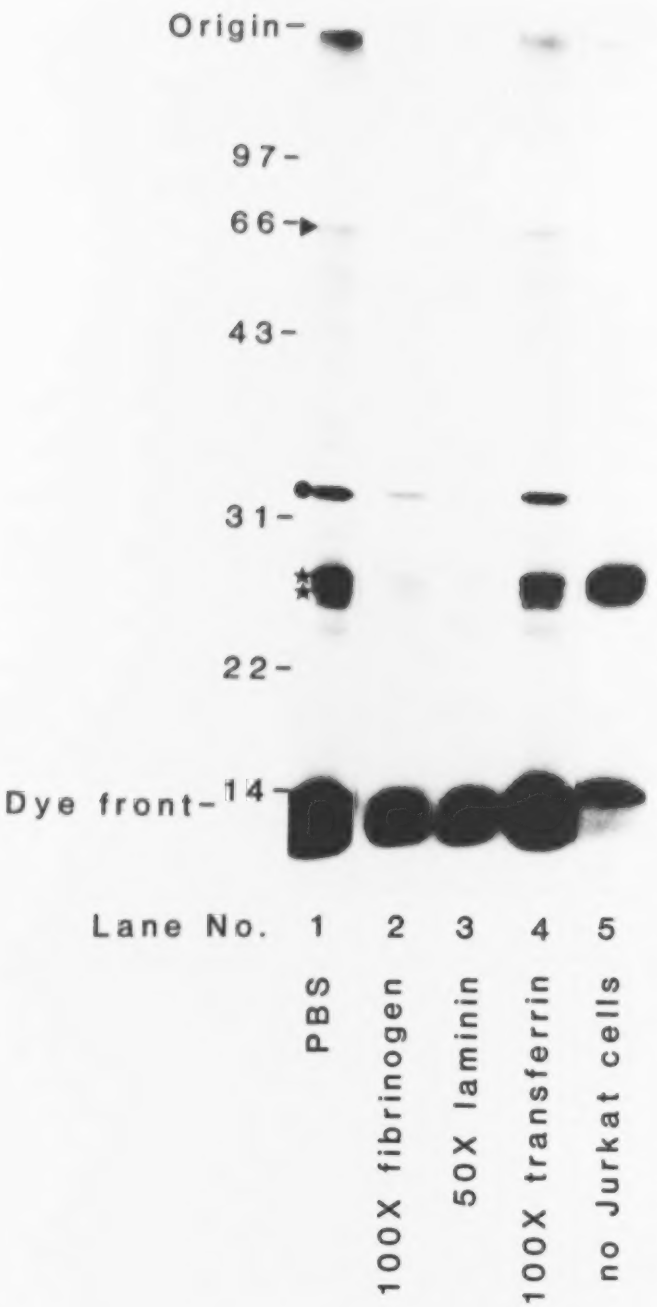

Origin-
97-
66-
43-
31-
22-
14-
Dye front-
Lane No. 1 2 3 4 5
PBS
100X fibrinogen
50X laminin
100X transferrin
no Jurkat cells

Figure IV.4

Inhibition by SNA and MAL of photoaffinity labeling of Jurkat cells using ^{125}I-APD-PT. Jurkat cells were photoaffinity labeled in the presence of these lectins by the procedure outlined in Materials and Methods, then were analyzed by SDS-PAGE and autoradiography as described in the legend to Figure IV.1. The positions to which the protein standards migrated are indicated on the left of each panel by a number representing their molecular weights (x 10^{-3}). The dye front and interface between the stacking and separating gels (origin) are also indicated in the figure. The 67, 50, and 32 kDa proteins are labeled with arrowheads, while autolabeled PT subunits are labeled with filled circles. The stars identify proteins of unknown origin that co-migrated with bands found in the lane containing ^{125}I-APD-PT treated with UV light in the absence of cells. The information in this figure was included as "data not shown" in the submitted manuscript.

Origin
97
66
43
31
22
Dye front
14
Lane No. 1 2 3
PBS
1000X SNA
1000X MAL

Figure IV.5

Two-dimensional SDS-PAGE analysis of PBMC (a) and Jurkat cell (b) proteins (arrows) photoaffinity labeled with ^{125}I-ASD-PT. Photoaffinity labeling and two-dimensional SDS-PAGE were performed as described in the Materials and Methods section. Samples were analyzed in the SDS-PAGE dimension in the presence of 50 mM DTT on 12.5% acrylamide separating gels. The autoradiogram used to produce the figure was prepared as described in the legend to Figure IV.1. The pH gradient determined from duplicate IEF gels is indicated at the bottom of the figure. The positions to which the protein standards migrated in the SDS-PAGE dimension are indicated (molecular weights x 10^{-3}) on the left of each panel.

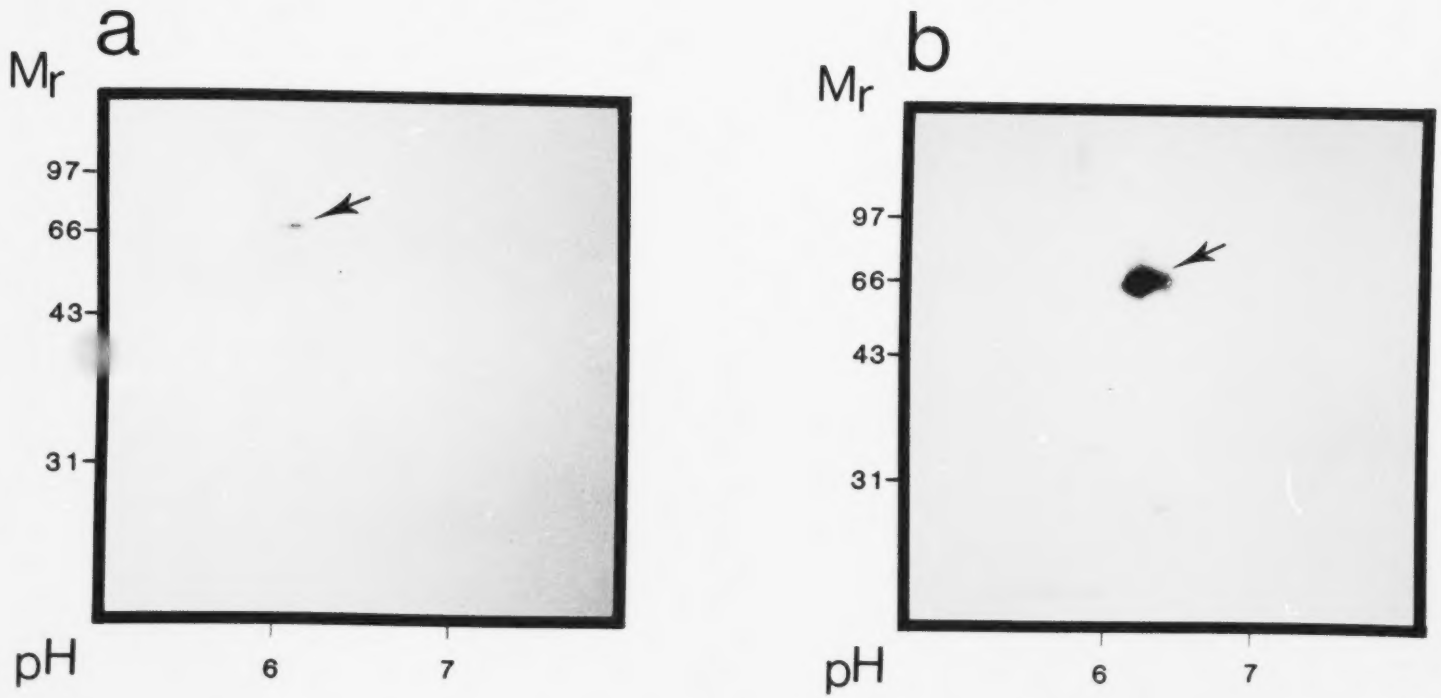

a
Mr
97
66
43
31
pH
6
7
b
Mr
97
66
43
31
pH
6
7

Figure IV.6

SDS-PAGE analysis of Jurkat cells photoaffinity labeled with ^{125}I-ASD-LPS (panel a) and ^{125}I-ASD-PT (panel b). Jurkat cells were photoaffinity labeled as described in the Materials and Methods section in the presence or absence of underivatized PT or LPS as indicated at the bottom of the figure. The autoradiograms used to produce the figure were prepared as described in the legend to Figure IV.1. The positions to which the protein standards migrated are indicated (molecular weights x 10^{-3}) on the left of the panel.

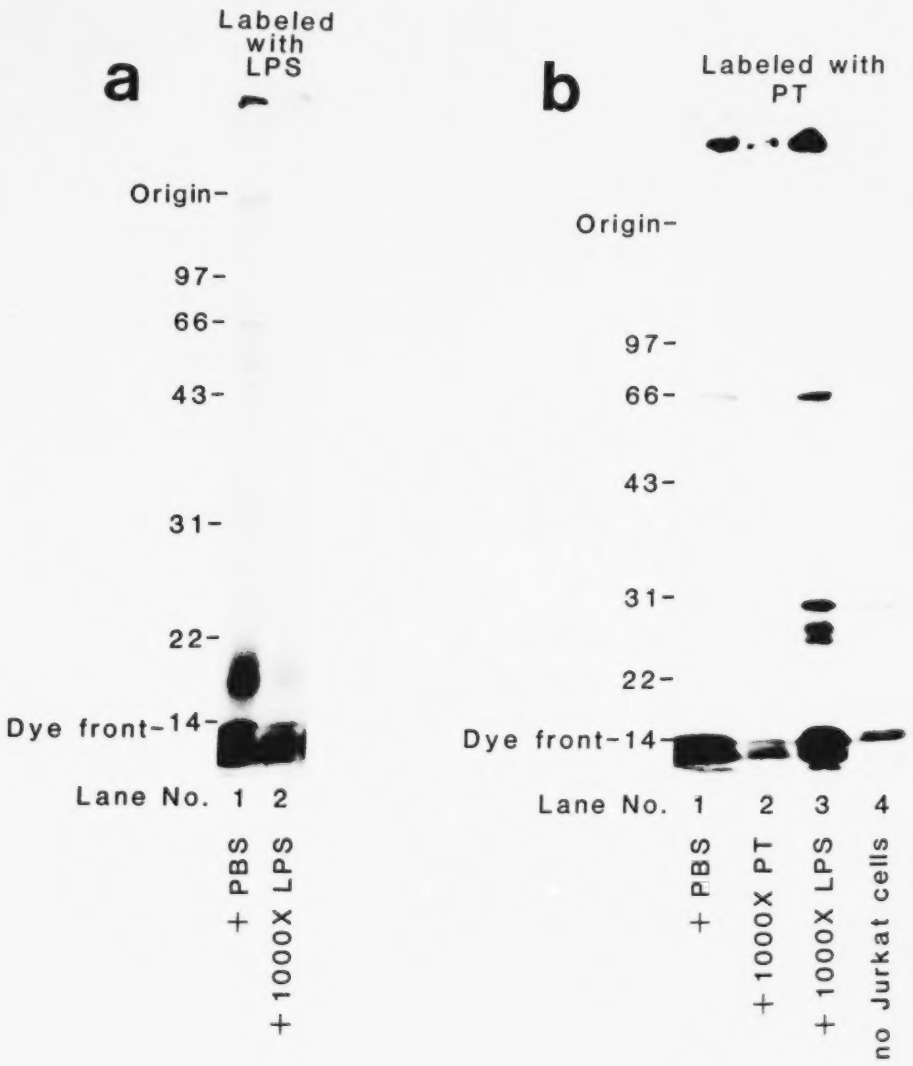
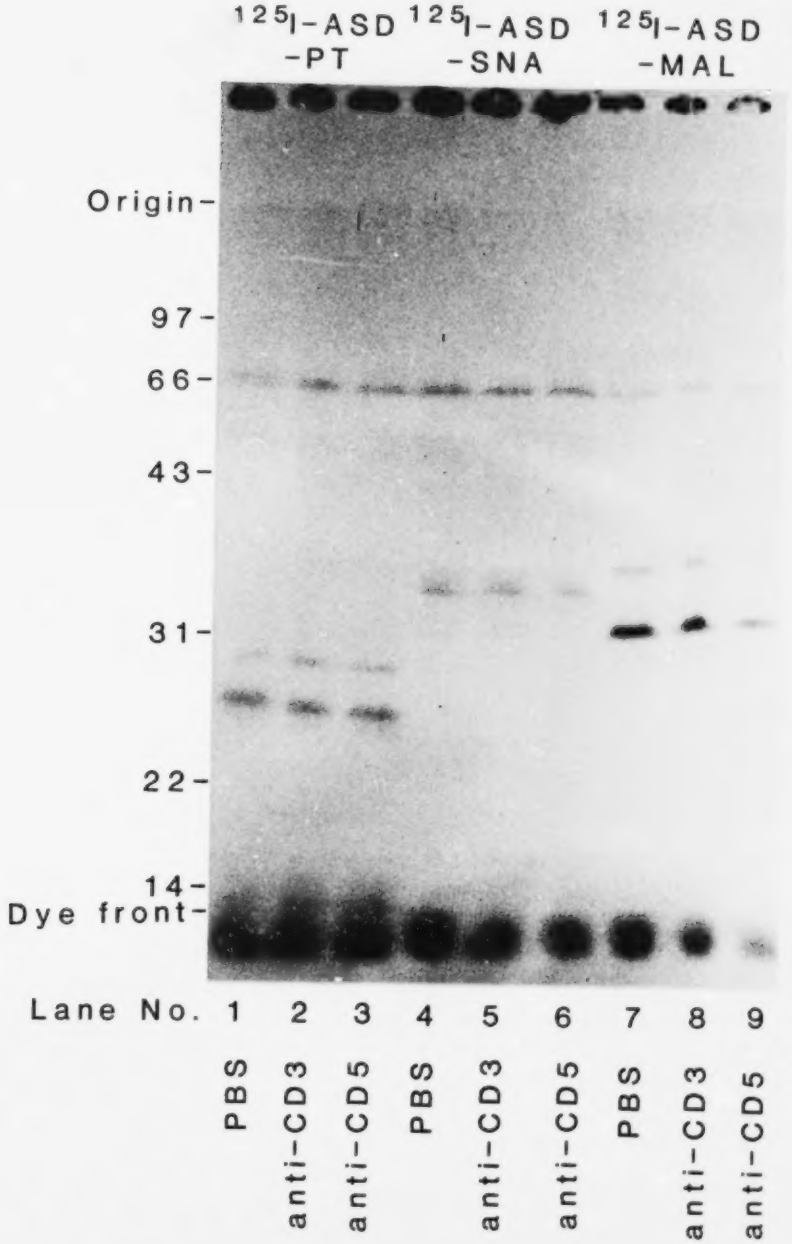

a
Labeled with LPS
Origin-
97-
66-
43-
31-
22-
Dye front-14-
Lane No. 1 2
+ PBS
+ 1000X LPS
b
Labeled with PT
Origin-
97-
66-
43-
31-
22-
Dye front-14-
Lane No. 1 2 3 4
+ PBS
+ 1000X PT
+ 1000X LPS
no Jurkat cells

Figure IV.7

Effect of antibodies to T cell antigens on photoaffinity labeling of Jurkat cells by ^{125}I-ASD-PT. Jurkat cells were photoaffinity labeled as described in the Materials and Methods section in the presence of excess anti-CD5, anti-CD3, or PBS alone, as indicated at the bottom of the figure. The autoradiograms used to produce the figure were prepared as described in the legend to Figure IV.1. The positions to which the protein standards migrated are indicated (molecular weights x 10^{-3}) on the left of the panel. The information in this figure was included as "data not shown" in the submitted manuscript.


^{125}I-ASD -PT
^{125}I-ASD -SNA
^{125}I-ASD -MAL
Origin
97
66
43
31
22
14
Dye front
Lane No. 1 2 3 4 5 6 7 8 9
PBS
anti-CD3
anti-CD5
PBS
anti-CD3
anti-CD5
PBS
anti-CD3
anti-CD5

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PREFACE TO CHAPTER V

Though we had identified several potential receptors for PT on Jurkat cells, we did not know whether PT bound each independently or whether the various proteins acted as a functional complex. To answer this question, we needed to first solubilize protein complexes from Jurkat cell plasma membranes, then to selectively isolate and identify PT-receptors using an affinity isolation procedure such as immunoprecipitation. We were concerned that immunoprecipitation of PT-receptor complexes would involve too many labile interactions for efficient recovery of receptors, since PT bound to receptors would be precipitated by antibody specific for PT and immobilized protein A-agarose. Additionally, the anti-PT antibodies available to us did not exhibit good binding to PT associated with glycoproteins, raising questions as to whether they would recognize PT bound to plasma membrane receptors.

Biotin-conjugated ligands have been used successfully to isolate receptors for transferrin in *Neisseria meningitidis* and *Pasteurella hemolytica* (1,2). We therefore decided to synthesize a biotinylated derivative of PT for use in studies of PT receptors and PT binding. PT-biotin associated with solubilized receptors could then be removed from solution using immobilized streptavidin, which binds biotin with high affinity. PT-biotin could also be used to determine whether glycoproteins were capable of competing for PT binding to receptors in intact cells (see section I.C.4) as well in ELISA binding assays designed to elucidate the relative binding affinities of PT for different glycoproteins.

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PREFACE TO CHAPTER VI

The 70 kDa protein photoaffinity labeled by ^{125}I-ASD-PT, -MAL, -SNA, and -LPS, co-migrates on SDS-PAGE with bovine and human serum albumin. During the course of the experiments described above, I noticed that when photoaffinity labeling of Jurkat cells was done in the presence of FBS or BSA, the 70 kDa protein was not labeled. Despite the fact that the amount of cell-bound, autolabeled PT was unchanged in these experiments, I became concerned that the high concentrations of BSA may be competing for PT binding to a subset of BSA molecules tightly associated with the cell surface rather than an endogenously synthesized Jurkat cell receptor. Presumably HSA, which is functionally homologous to BSA, could substitute for BSA as either the competitor or the absorbed receptor.

Several lines of evidence suggested that the 70 kDa PT-binding protein from Jurkat cells and T lymphocytes was not BSA or HSA. First, neither BSA nor HSA were detected in immunoblots of Jurkat cells (Chapter III, Figure III.6). This technique is relatively insensitive, however, and may not have detected small amounts of serum albumin absorbed onto cell surfaces. The detection of the 70 kDa protein in unfractionated PBMC that had never encountered FBS indicated that this protein was not BSA or fetuin, though the possibility remained that it was HSA from human serum (Chapter III, Figure III.5). Furthermore, serum albumins are not known to contain *N*-linked oligosaccharides. ^{125}I-ASD-SNA and ^{125}I-ASD-MAL, both sialic acid specific lectins, photoaffinity labeled the 70 kDa protein (Chapter IV, Figure IV.1). It remains possible, though, that PT, SNA, and MAL associated with serum albumins through protein-protein interactions.

I therefore set out to investigate the interaction between serum albumin and PT with an ELISA technique that had previously been used to quantitate the relative affinity of

PT binding to various glycoproteins (Chapter V, Table V.1). In addition, I decided to investigate the possibility that serum albumin may be binding to ASD rather than PT. The occurrence of this type of association would be consistent with the reduction of photoaffinity labeling of the 70 kDa PT receptor by serum albumin and the lack of effect of this protein on the association of autolabeled PT with toxin receptors on Jurkat cells. It would also support the hypothesis that the 70 kDa PT-receptor on Jurkat cells is produced by the cell rather than absorbed from the medium.

CHAPTER VI

BOVINE SERUM ALBUMIN BINDS PHOTOAFFINITY CROSSLINKING AGENTS[1]

A. INTRODUCTION

Photoaffinity labeling techniques using heterobifunctional, cleavable, iodinatable crosslinking agents have proven to be useful for investigating receptor-ligand interactions (2,5,7,9). A major advantage of these procedures is that the architecture of the plasma membrane bearing specific receptors is maintained. Only receptors normally available for interaction with the ligand under study will therefore be able to react with the photoaffinity probe. The photoaffinity labeling procedure is summarized in Figure III.2, Chapter III. The ligand (which may be protein, peptide, or LPS) is first covalently modified with an iodinated crosslinking agent such as sulfosuccinimidyl 2-(*p*-azidosalicylamido)ethyl-1,3′-dithiopropionate (SASD) or N-[4-(*p*-azidosalicylamido)butyl]-3′(2′-pyridyldithio)propionamide (APDP). After the biological activity of the resulting photoaffinity probe is confirmed, the complex is allowed to interact non-covalently with its receptor on whole cells. Activation of the azido group of the crosslinker using ultraviolet light then catalyzes the covalent insertion of the crosslinker into carbon-carbon or carbon-hydrogen bonds in the receptor (6). The products of the reaction are subsequently analyzed using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) in the presence of dithiothreitol. The disulfide bond within the crosslinker is reduced, resulting in the separation of the ligand from the receptor and leaving a small radioactive tag on the

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[1] The information presented in this chapter has also been prepared for publication.

receptor molecule. Receptors can then be identified using autoradiography.

We have successfully used the photoaffinity labeling technique to identify pertussis toxin (PT) receptors on T lymphocytes and Jurkat cells (2; Chapters 3 and 4). When thoroughly washed cells were used for these experiments, we identified a 67 to 70 kDa PT receptor in both cell types using SASD-modified PT. Two additional PT-receptors having apparent molecular weights of approximately 50 and 32 kDa were labeled when APDP-modified PT was used (Chapter IV). However, in the course of our investigations, we noticed that the inclusion of bovine serum albumin (BSA) in the reaction buffer resulted in the complete abrogation of photoaffinity labeling of the cell-surface receptors. BSA did not appear to affect the interaction of PT with its receptor(s), but instead appeared to bind specifically to the crosslinking agent, SASD. We therefore decided to further investigate the mechanism by which BSA interferes with photoaffinity labeling reactions.

B.RESULTS AND DISCUSSION

The protocols used for making ^{125}I-ASD-PT and photoaffinity labeling Jurkat cells were the similar to those reported previously (2; Chapter IV), with the exception that labeling was performed in the presence and absence of BSA. As seen in Figure VI.1, excess BSA completely abrogated photoaffinity labeling of the 67 to 70 kDa protein in Jurkat cells, though it did not appear to affect the amount of autolabeled PT bound to cells. The presence of BSA therefore did not appear to affect binding of ^{125}I-ASD-PT to Jurkat cells per se. This raised two possibilities: 1) BSA binds directly to PT by a mechanism distinct from PT binding to cells or 2) BSA interacts directly with ASD. Since inhibition of photoaffinity labeling was essentially complete at 100 times excess of BSA over ^{125}I-ASD-PT, we speculated that the

interaction of BSA with ASD or PT was specific and of high affinity.

We have been unable to demonstrate specific binding of PT-biotin to human serum albumin (HSA), which is structurally and functionally analogous to BSA. Microtitre plates coated with 100 µl volumes of HSA (50 µg/ml) and fetuin (5 µg/ml) were probed with 0.4 µg (20 µl) of PT-biotin as described previously (3; Chapter V). Wells used for negative controls were treated in exactly the same way as those containing HSA and fetuin, except that no protein was added to the coating buffer. The color development in HSA-coated and negative control wells was 9.91 ± 3.51% (n = 10) and 9.68 ± 2.74%, respectively, of the color development in fetuin-coated wells. PT-biotin, therefore, did not bind specifically to HSA. BSA appears to behave in a similar way, since the addition of BSA to coated microtitre plates as a blocking agent did not interfere with the specific interaction of PT-biotin with known glycoprotein substrates for PT (3; Chapter V). Since PT does not bind specifically to HSA or BSA, therefore, it must be interacting with the crosslinker.

To test our hypothesis, we have investigated the interaction of SASD with BSA. SASD was first dissolved in 0.1 M Tris-HCl, pH 8, containing 5% dimethylsulfoxide. This buffer was used to promote the reaction of SASD with amino groups in the Tris-HCl as well as the hydrolysis of succinimidyl groups from SASD, thereby preventing the covalent modification of lysines in BSA by the crosslinking agent. A 10 µl aliquot of SASD (10 µg) was iodinated for 1 min with 3.7 MBq of Na[^{125}I] in a 12 by 75 mm culture tube coated with IODO-GEN. The ^{125}I-SASD was then placed in a clean, foil-wrapped 12 by 75 mm culture tube, and incubated for 1 h to allow hydrolysis of the succinimidyl group.

Unlabeled SASD was also incubated for 1 h in Tris-HCl, pH 8, to allow hydrolysis of the succinimidyl groups and formation of ASD. BSA (20 µg in 20 µl) was incubated for 30 min with ASD or with Tris-HCl, pH 8, alone. The iodinated,

cleaved ^{125}I-ASD was then added to these mixtures and allowed to associate with the BSA for 30 min on ice. Next, the BSA/^{125}I-ASD complexes were separated from unbound ^{125}I-ASD and $^{125}I_2$ using a 10 ml volume (19.5 by 0.8 cm) Sephadex G-25 column pretreated with 0.5 ml of 0.2% blocking reagent (hydrolyzed gelatin; Boehringer Mannheim) in PBS. Column fractions (0.5 ml) were collected with PBS and counted in a LKB RackGamma II gamma counter. Portions (50 µl) of the void volume fraction containing the highest activity of BSA-associated ^{125}I-ASD were used for SDS-PAGE analysis.

^{125}I-ASD co-eluted with BSA at the void volume of the Sephadex G-25 columns (Figure VI.2A). The input counts of Na^{125}I were approximately 7 x 10^7 cpm for each experiment and the total counts in void volume fractions represented 0.48%, 0.27%, and 0.25% of the input counts for experiments with no ASD, 10x ASD, and 20x ASD, respectively. Although the reaction mixtures had not been exposed to light, 92 ± 2.9% (n = 11) of the radioactivity in the G-25 void volume fractions was precipitated with trichloroacetic acid. Unlabeled ASD effectively competed for ^{125}I-ASD binding to BSA as demonstrated by a significant reduction in counts co-eluting with BSA in the void volume of the Sephadex G-25 columns (Figure VI.2A). Since ^{125}I-ASD had been pre-incubated with Tris-HCl, it was unlikely that the crosslinking reagent had interacted with free amino groups in BSA. Nonetheless, SDS-PAGE analysis (Figure VI.2B) of G-25 void volume fractions in the presence of dithiothreitol suggested that ASD had become covalently attached to the BSA.

^{125}I-ASD did not associate with transferrin or phosvitin (Figure VI.3B). The amount of ^{125}I-ASD eluting with phosvitin in the Sephadex G-25 column void volume fractions was similar to the amount eluting in the same position when ^{125}I-ASD was incubated with buffer only (Figure VI.3A), indicating that there was little interaction between ^{125}I-ASD and phosvitin. There was a slight association of ^{125}I-ASD with transferrin, possibly due to the fact that transferrin is a serum protein

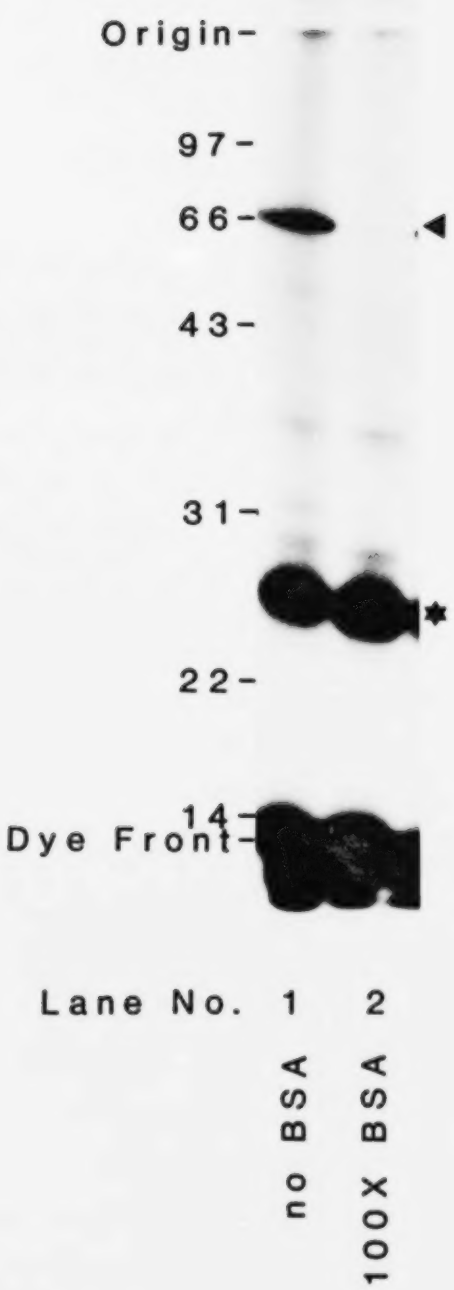
and may have a capacity for hydrophobic interactions with lipids. Transferrin is known to interact with LPS (1). The presence of a void volume peak containing ^{125}I-ASD in the absence of added protein may represent an interaction of the labeled crosslinking agent with gelatin peptides from the Boehringer Mannheim blocking reagent. These peptides can be seen in Figure VI.2B, lane 1 (stars).

BSA binds fatty acids (8), octanoate, and tryptophan (4). It appeared that ASD may have been interacting with the domain of BSA responsible for binding hydrophobic organic compounds. To investigate this possibility, we determined the ability of SDS and phosphatidylethanolamine to compete with ^{125}I-ASD for binding to BSA. The association of ^{125}I-ASD with BSA was reduced with both SDS and phosphatidylethanolamine (Figure VI.3C). The fact that low concentrations of phosphatidylethanolamine competed with the ^{125}I-ASD strongly suggests that both molecules were associating with the fatty acid binding sites on BSA. Furthermore, since SDS was capable of interfering with the interaction of ^{125}I-ASD and BSA, the labeled BSA visualized by SDS-PAGE in Figure VI.2B must have contained covalently attached ^{125}I-ASD.

Together, these results suggest that the presence of BSA, or fetal bovine serum containing BSA, may inhibit the photoaffinity labeling reaction mediated by crosslinking agents such as SASD. Photoaffinity labeling protocols should therefore be devised in such a way as to eliminate BSA, HSA, and other proteins that act as lipid carrier molecules.

Figure VI.1

Photoaffinity labeling of Jurkat cells with ^{125}I-ASD-PT in the absence (lane 1) and presence (lane 2) of 100 times molar excess of BSA. Jurkat cells were photoaffinity labeled with ^{125}I-ASD-PT, solubilized in SDS-PAGE sample buffer containing 50 mM dithiothreitol, and analyzed on a 12.5% separating gel. The gel was then stained, dried, and exposed to X-ray film to produce the autoradiogram presented in the figure. The positions to which the protein standards migrated are indicated on the left of the panel by numbers representing their molecular weights (x 10^{-3}). The 70 kDa Jurkat cell-surface protein is labeled with an arrowhead, and the 24 kDa band representing PT's S2 and S3 subunits is designated with a star.


Origin-
97-
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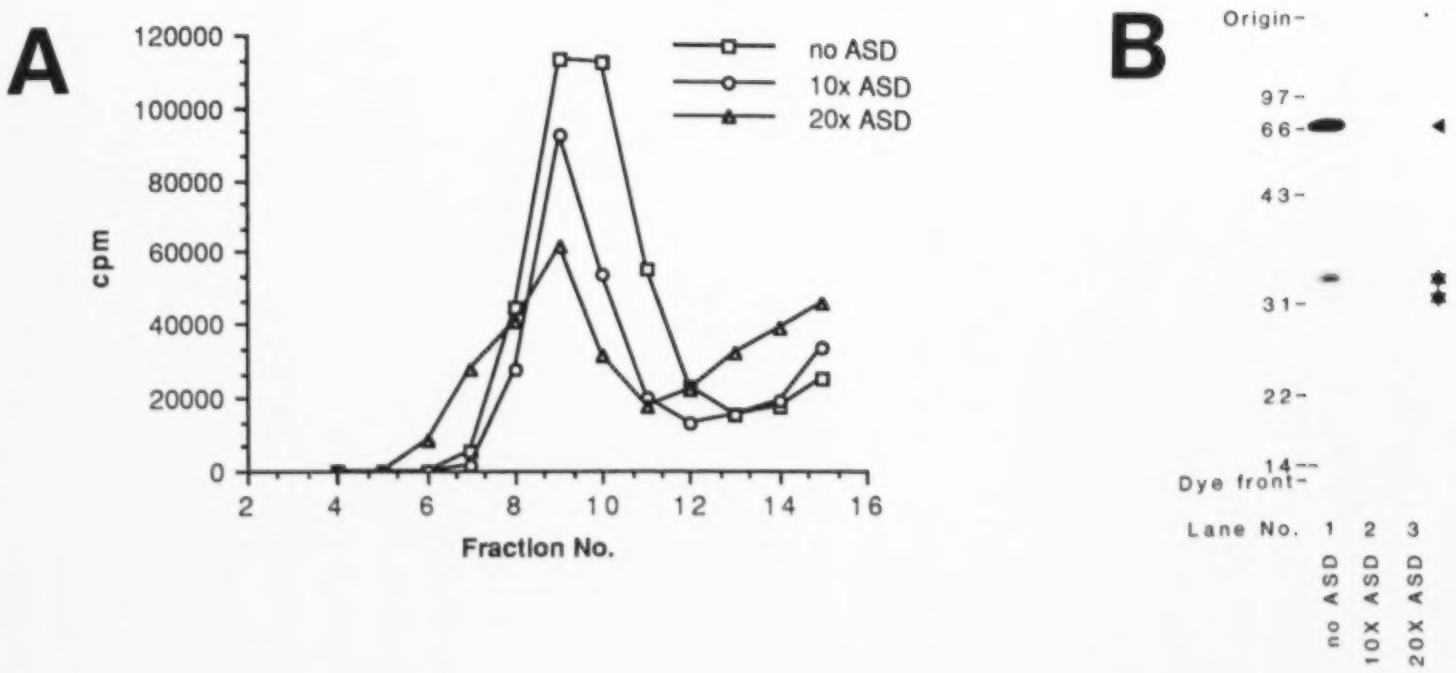
43-
31-
22-
14-
Dye Front-
Lane No. 1 2
no BSA
100X BSA

Figure VI.2

Association of ^{125}I-ASD with BSA. A. Co-elution of ^{125}I-ASD and BSA from G-25 columns. BSA and ^{125}I-ASD were incubated together in the presence or absence of 10 and 20 times excess ASD. These mixtures were applied to G-25 columns and 0.5 ml fractions were eluted from the columns with PBS. B. SDS-PAGE and autoradiography of the peak fractions obtained from the experiments shown in Figure 2A. 50 µl of each peak fraction was dissolved in sample buffer with 50 mM dithiothreitol and analyzed on a 12.5% SDS-PAGE gel. The autoradiogram used to produce the figure was prepared as described in the legend to Figure 1. BSA (shown by the arrowhead) was incubated with ^{125}I-ASD in the presence of buffer alone (lane 1), 10X ASD (lane 2), and 20X ASD (lane 3). The amount of BSA present in all lanes of the stained gel was approximately the same. The positions to which the protein standards migrated are indicated on the left of the panel by numbers representing their molecular weights (x 10^{-3}).


A
120000
100000
80000
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8
10
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14
16
Fraction No.
no ASD
10x ASD
20x ASD
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Origin
97
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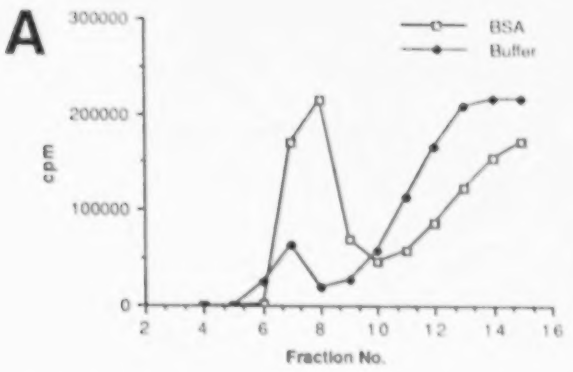
31
22
14
Dye front
Lane No. 1 2 3
no ASD
10X ASD
20X ASD

Figure VI.3

Gel filtration of ^{125}I-ASD in the absence and presence of proteins and inhibitors. A. Elution of ^{125}I-ASD from columns after incubation with either BSA (20 µg) or buffer. Column chromatography and analysis of the fractions was performed as shown in the legend to Figure 2. B. Co-elution of ^{125}I-ASD with BSA, transferrin, and phosvitin. The ^{125}I-ASD was incubated with 20 µg BSA, 20 µg transferrin, and 40 µg phosvitin, then subjected to column chromatography on Sephadex G-25 columns and analyzed as detailed in the text and in Figure VI.2a. C. Effects of SDS and phosphatidylethanolamine on the co-elution of ^{125}I-ASD and BSA from Sephadex G-25 columns. BSA (20 µg) was incubated with 0.1% SDS or 20 ng phosphatidylethanolamine. ^{125}I-ASD (equivalent to 10 µg of unhydrolyzed SASD) was then added to these mixtures and allowed to associate with the BSA according to the protocol in the text. Column chromatography and analysis of the fractions was performed as shown in the legend to Figure VI.2.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200000
100000
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BSA
Buffer
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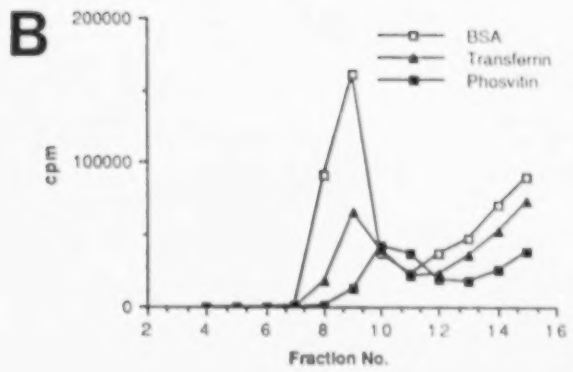
4
6
8
10
12
14
16
Fraction No.


B
200000
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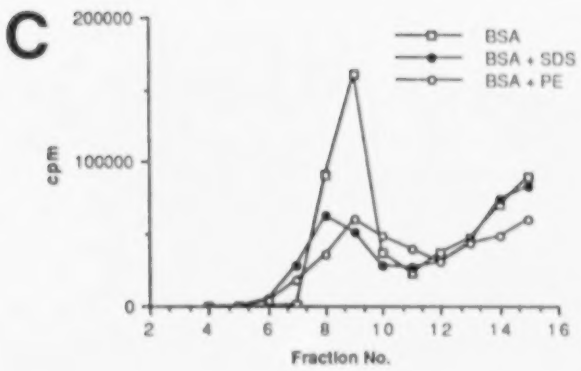
cpm
BSA
Transferrin
Phosvitin
2
4
6
8
10
12
14
16
Fraction No.


C
200000
100000
0
cpm
BSA
BSA + SDS
BSA + PE
2
4
6
8
10
12
14
16
Fraction No.

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CHAPTER VII

GENERAL DISCUSSION AND CONCLUSIONS

A. Summary.

At the beginning of this project, very little was known about eukaryotic receptors for PT. Studies of the toxin's interaction with glycoproteins and of oligosaccharide structures required for PT binding had provided tantalizing clues about the nature of these receptors. It was not known, however, whether PT bound to cellular receptors by mechanisms similar to those involved with (lectin-like) glycoprotein binding or if additional (protein-protein or electrostatic) interactions might also be involved.

The isolation of a relatively large number of PT-binding proteins from goose erythrocytes and T lymphocytes by affinity chromatography supported the theory that PT is capable of associating with plasma membrane proteins in different ways (see Chapters II and III). Some proteins appeared to bind PT mainly through electrostatic interactions, since washing PT-agarose columns with buffer containing 1 M NaCl reduced the number of proteins bound. Other proteins specifically associated with PT by a process that was not sensitive to high salt and which could be inhibited by iodination of the toxin. Using this method, however, it was not possible to determine whether PT was capable of associating with these same proteins on intact cells.

To identify proteins that were responsible for PT binding to intact cells, I employed a photoaffinity labeling technique using the crosslinkers, SASD and APDP (see Chapters III and IV). Three Jurkat cell proteins with approximate molecular masses of 70, 50, and 32 kDa were specifically labeled with ^{125}I-APD-PT, while only the 70 kDa protein was labeled consistently with ^{125}I-ASD-PT. These differences in labeling were thought to result either from different spatial

distributions of the crosslinkers on the surface of PT or from inactivation of a binding site in the toxin when the amino-reactive SASD was used. The 70 kDa PT-binding protein identified on Jurkat cells was also found on T lymphocytes.

^{125}I-ASD-LPS photoaffinity labeled Jurkat cell proteins with sizes similar to those labeled by ^{125}I-APD-PT (see Chapter IV). The 70 kDa Jurkat cell protein labeled by ^{125}I-ASD-PT had the same isoelectric point as the 73 kDa protein labeled by ^{125}I-ASD-LPS, supporting the hypothesis that PT and LPS may associate with the same receptor on these cells. LPS did not appear to compete for photoaffinity labeling of the 70 kDa protein by ^{125}I-ASD-PT, however, indicating either that LPS and PT bind to different sites on the same protein or that they bind to two different proteins with very similar sizes and isoelectric points.

The sialic acid-specific lectins, SNA and MAL, reduced the labeling by ^{125}I-ASD-PT and ^{125}I-APD-PT of the 70, 50, and 32 kDa Jurkat cell proteins (see Chapter IV). Three proteins with the same apparent molecular weights were photoaffinity labeled by ^{125}I-ASD- and ^{125}I-APD-modified SNA and MAL. This labeling was eliminated by unmodified PT, indicating that the lectins and PT recognized oligosaccharide binding epitopes on the labeled proteins. Finally, several glycoproteins reduced the labeling of all three Jurkat cell proteins by PT, MAL, and SNA. These data provided the first evidence that PT binds to eukaryotic cell receptors through lectin-like interactions. Furthermore, the observation that PT, lectins, and LPS all appear to photoaffinity label the same proteins on Jurkat cells suggests that these proteins may be receptors for ligands that are important for normal cell function. The 73 kDa LPS receptor has previously been characterized as a glycoprotein that is involved with the induction of macrophage-mediated tumor cytotoxicity (1,4), supporting the idea that the 70 kDa protein labeled by PT, MAL, and SNA may also be a functional receptor. The common identity of the receptors for these various ligands needs to be further

investigated through peptide mapping of the labeled proteins.

From the outset of my photoaffinity labeling investigations, I was acutely concerned with the possibility that the 70 kDa receptor was a serum component absorbed onto the surface of cells. The major component of serum is albumin, which co-migrated with the 70 kDa receptor on SDS-PAGE. Therefore, a major emphasis was placed on determining whether or not the 70 kDa receptor was or was not serum albumin. Several lines of evidence indicated that the labeled protein was not serum albumin absorbed onto the cell surface. SNA and MAL, which bind only to sialic acid-containing glycoproteins, also labeled this protein (Chapter IV). Since BSA and HSA are not glycoproteins, it is highly unlikely that the 70 kDa protein is serum albumin. The 70 kDa protein was not detected by antibodies specific for BSA or HSA in blots containing Jurkat cell proteins (Chapter III). Furthermore, I was unable to demonstrate binding of PT-biotin to HSA in an ELISA assay, though PT bound fetuin with high affinity under the same conditions (Chapter VI).

Despite this evidence, it was not possible to totally eliminate serum albumin as a receptor candidate. The assays discussed above may not have been sensitive enough to detect low levels of albumin. Therefore, a series of experiments were performed to determine whether ^{125}I-ASD-PT was capable of interacting with BSA. These experiments were predicated on the knowledge that serum ablumin contains lipid binding domains that could bind to the hydrophobic ASD moiety on derivatized PT. Low concentrations of BSA completely eliminated photoaffinity labeling of Jurkat cell proteins when BSA was included in the crosslinking buffer, though it did not appear to affect the amount of autolabeled ^{125}I-ASD-PT associated with the cells (see Chapter VI). Hydrolyzed SASD associated specifically with BSA, but not with transferrin or phosvitin. As predicted, the interaction of ASD with BSA was disrupted by SDS and phosphatidylethanolamine, suggesting that ASD interacts with binding sites for hydrophobic

molecules in BSA. It is likely, therefore, that BSA interferes with photoaffinity labeling of Jurkat cell proteins by binding directly to the ^{125}I-ASD linked to PT.

While these experiments demonstrated that ASD could interact specifically with BSA, they did not address the problem of whether the presence of the 70 kDa receptor on Jurkat cells was the result of absorption of serum albumin onto cell surfaces. To address this problem and determine the identity of the 70 kDa receptor, proteins from ^{125}I-APD-PT-labeled cells were blotted to polyvinyldifluoride (PVDF) membranes. These membranes were stained and analyzed by autoradiography, and the labeled 32, 50, and 70 kDa proteins were subjected to *N*-terminal amino acid microsequencing. Though the sequence of a BSA standard obtained from the same blot corresponded with the published sequence, no information could be collected for the three labeled Jurkat cell proteins. Since the Coomassie blue-stained bands corresponding to these proteins were readily observable, the failure to obtain sequences for these proteins was probably the result of blockage of the *N*-terminal amino acid. By this criterion, all three proteins appeared to be eukaryotic proteins produced by the Jurkat cells. From the preceding discussion, it is obvious that more work needs to be done to determine the identity of the 70 kDa receptor. One way the albumin issue may be resolved would be to peptide map the labeled receptor protein and BSA. In preliminary experiments, peptide maps were generated by proteinase K digestion of the 70 kDa protein photoaffinity labeled by ^{125}I-ASD-PT and ^{125}I-ASD-LPS. Two major peptides with molecular weights of approximately 37 and 23 kDa appeared to be identical in SDS-PAGE gels. Proteinase K digestion of photoaffinity labeled BSA also produced peptides with molecular weights of 37 and 23 kDa. These results suggested that the receptors for PT and LPS are very similar molecules. The results also supply additional proof that the major 70 kDa PT and LPS receptor

may be BSA or a closely related molecule (manuscript submitted for publication).

CD5 is a 67 kDa antigen found on T lymphocytes and a subset of B lymphocytes. Because this antigen was approximately the same size as the protein labeled by ^{125}I-ASD-PT, I designed experiments to determine whether the two proteins were identical. The 70 kDa PT receptor was not immunoprecipitated by antiserum specific for CD5, and this antiserum did not competetively reduce the labeling of this protein by ^{125}I-ASD-PT (Chapter IV). It is unlikely, therefore, that the 70 kDa protein is CD5.

B. Possible identity of the PT receptors on various cell types.

Relatively low concentrations of PT are capable of affecting many cell types from different species (see Chapter I). This implies that these cells have high affinity receptors for PT and has important consequences for the understanding of the role of PT in the disease process, the effects of PT in pertussis vaccines, and the use of PT as a tool in pharmacological research. It is possible that each type of cell has a unique PT receptor, though it seems more likely that a single protein or family of proteins may be responsible for PT binding to many of the cells discussed in Chapter I. The possibility that the 70 kDa PT receptor may be identical to the 73 kDa LPS- and peptidoglycan-receptor (Chapter IV), coupled with the fact that the 73 kDa LPS and peptidoglycan receptor is found on a number of cell types (8,11,16), supports this hypothesis. If a single subset of cell-surface proteins is indeed responsible for binding and responding to all these biologically important modulators of cell function, it would be expected to have important functions in the normal development and activity of the cells.

Such highly conserved proteins could belong to a family of receptors for cellular adhesion molecules such as selectins or laminin. The 67 kDa laminin receptor and 32 kDa Mac-2 antigen, in particular, have many properties in common with the PT receptor we have identified. The 67 kDa laminin binding protein has an apparent molecular weight ranging from 67 to 72 kDa, depending on the cell line used for study, and additionally has a pI of 6.4 ± 0.2 (19). These values for both molecular weight and pI are shared by the receptors for PT, LPS, and peptidoglycan (7,8) see Chapter IV, above). Both the Mac-2 antigen and the 67 kDa laminin binding protein are found on a number of cell types (12,15,18,19) including a variety of lymphoid and myeloid cells (9). The 67 kDa laminin receptor, in common with our 70 kDa PT receptor, has a blocked amino terminus (12). In addition, it may be identical to the 67 kDa elastin receptor (10,13,14). Lymphocytes have much lower numbers of the 67 kDa laminin receptor (~3,000 sites per cell) than HL 60 cells (~8,500 sites per cell) or granulocytes (~25,000 sites per cell) (9).

Sobel and colleagues found that the 67 kDa laminin receptor is a chimeric molecule composed of a well-characterized 37 kDa polypeptide plus a second polypeptide of unknown origin that belongs to the β-galactoside lectin family (2,3). Importantly, Mercurio and colleagues (20) have reported that the Mac-2 antigen (also known as carbohydrate-binding protein 35, RL-29, L-34, and low affinity IgE-binding protein) can form either functional homodimers or heterodimers with an uncharacterized protein. Because the Mac-2 antigen is a galactose-specific laminin-binding lectin that has similar specificity and activity to the 67 kDa laminin receptor (5,15,20), it is a potential candidate for the second peptide comprising the 67 kDa laminin receptor. If true, this could explain the appearance of both the 70 and 32 kDa proteins after labeling of Jurkat cells with ^{125}I-APD-PT.

I have recently done preliminary experiments designed to determine whether Mac-2 or the 67 kDa laminin receptor are

involved in PT binding to Jurkat cells. Photoaffinity labeled PT receptors from detergent-solubilized Jurkat cells could not be immunoprecipitated using rat anti-Mac-2 monoclonal antibodies. These antibodies also did not detect Mac-2 among Jurkat cell proteins blotted to nitrocellulose. However, this may reflect the limitations of the procedures as they were used rather than the absence of the Mac-2 antigen. It is often difficult to detect proteins on nitrocellulose using monoclonal antibodies, for instance. Factors such as the sensitivity, species, and isotype of antibody, as well as a small number of (labeled) receptors on cells can limit the utility of the immunoprecipitation technique. Finally, several attempts to isolate PT-binding complexes from Jurkat cell lysates using PT-biotin and immobilized streptavidin were also unsuccessful, suggesting that detergents may interfere with the specific binding of PT to its receptors.

The 67 kDa laminin/elastin receptor contains binding sites for peptide determinants as well as for lactose (3,12,14). The ligand affinity of this receptor is decreased in the presence of lactose (14), which is also capable of releasing the receptor from cell surfaces (10). In order to determine whether PT receptors are identical to the 67 kDa laminin-binding protein, I have treated photoaffinity labeled Jurkat cells with 100 mM lactose (in PBS) in an unsuccessful attempt to release the receptor from these cells. Two reasons for the lack of success may be the low efficiency of photoaffinity labeling (see Chapter III) coupled with low numbers of receptors on these cells. Disrupting the interaction of ^{125}I-ASD-PT with receptors using 100 mM lactose may provide a more productive approach.

Laminin peptides, specifically VGVAPG and YISGR, have been used to interfere with laminin binding to various receptors and to elute laminin receptors from laminin columns (12). The VGVAPG peptide is also homologous to the *N*-terminal sequence of PT's S3 subunit (VAPGIVI), raising the possibility that PT could bind to the 67 kDa laminin receptor

through protein-protein interaction. In preliminary experiments, however, I have been unable to compete for the photoaffinity labeling of PT receptors on Jurkat cells using VGVAPG, YISGR, or a synthetic peptide containing the first 14 amino acids from the amino terminus of the PT subunit S3.

Though the experiments described above are only preliminary, they indicate that the PT and laminin receptors may be different proteins. If this proves to be the case, it may be more productive to exploit the identity between the 67 kDa PT and LPS receptor(s). Morrison and colleagues (1,4) have described the production of monoclonal antibodies to both carbohydrate and protein determinants on the 73 kDa LPS receptor. These antibodies could be used to further explore the correspondence between PT and LPS receptors, as well as to obtain large enough quantities of these proteins for sequencing. Additionally, it may be possible to isolate larger quantities of labeled PT receptors using cell fractionation techniques similar to those described by Bright *et al.* (1).

Results from preliminary studies support the idea that the PT receptors identified on Jurkat cells and T lymphocytes may also be present on other cells. HeLa, Vero, HT 29, HEp 2, HuTu, CaCo2, and HL 60 cells all have proteins photoaffinity labeled by ^{125}I-ASD-PT that co-migrate in SDS-PAGE gels with the 32 kDa and 70 kDa Jurkat cell receptors. Other proteins also appear to be labeled in some of these cells, but the specificity of the interactions involved is not known, since competition experiments with unlabeled PT have not yet been performed. In addition to confirming the observations obtained with the cell types discussed here, it would be interesting to investigate PT receptors in many of the cell types and cell lines discussed in Chapter I to determine whether the 70, 50, and 32 kDa PT receptors are also found in these cells.

C. Expression and location of the 70 kDa PT receptor on Jurkat cells.

Experiments were designed to determine whether the 70 kDa PT receptor was an integral membrane protein or was less tightly associated with the plasma membrane of Jurkat cells. Non-integral membrane-associated proteins were removed using 0.5 M NaCl or 6 M guanidine hydrochloride before or after crosslinking the cells with ^{125}I-ASD-PT, producing the results shown in Figures VII.1 and VII.2. The addition of 6 M guanidine hydrochloride extracted most of the labeled protein from Jurkat cell membranes (Figure VII.1, lanes 3 and 4), while 0.5 M NaCl appeared to remove all of the labeled protein (Figure VII.2, lanes 1 and 2), suggesting that this protein may not be an integral membrane component. However, when Jurkat cells were first treated with 0.5 M NaCl, then photoaffinity labeled with ^{125}I-ASD-conjugated PT, SNA, MAL,or LPS, the 70 kDa protein was seen in autoradiograms (Figure VII.2). It was therefore not possible to completely remove all of this protein from membranes. The 32 kDa protein labeled by ^{125}I-APD-PT was also detected after treating cells with 0.5 M NaCl (Figure VII.2). These results suggest that the 70 kDa PT receptor is a peripheral protein tightly associated with plasma membranes of Jurkat cells and that the 32 kDa PT receptor may be an integral membrane protein or a more tightly associated peripheral membrane protein.

The 32 kDa protein was labeled on Jurkat cells that were grown in medium containing FBS and on Jurkat cells adapted to serum-free medium containing only transferrin and insulin (Figure VII.3), suggesting that this protein is endogenously synthesized by the cells. In this experiment, the 70 kDa PT receptor was also labeled on cells grown in serum free medium. Furthermore, labeling of both the 32 and 70 kDa PT receptors on cells grown in serum free medium was reduced by only a 100 times excess underivatized PT (Figure VII.3, lanes 3 and 4). This suggests that the labeling of both proteins results from the specific, high affinity

binding of PT to the Jurkat cell receptors. It is not clear why subsequent experiments using serum free cells failed to demonstrate labeling of the 70 kDa protein by ^{125}I-ASD- or ^{125}I-APD-PT. The results from one experiment suggested that the labeling of the 70 kDa protein on Jurkat cells grown without FBS was dependent on the subsequent addition of FBS to the cells. This indicated that the 70 kDa protein may have been absorbed from the medium and implied that it might be serum albumin, despite the evidence to the contrary (Chapter VII.A). Alternately, the process of adapting Jurkat cells to grow in serum free medium may have caused the selection of variants defective in the production of this protein. It is also possible that the adaptation to growth without serum resulted in the down-regulation of some plasma membrane components, including the 70 kDa protein. Similarly, something in the medium may have been necessary for induction of the synthesis, translocation, or increased exposure of a 70 kDa plasma membrane protein in these cells. Incubation of Jurkat cells for 24 h in serum free medium resulted in a large degrease in the intensity of the photoaffinity labeled 70 kDa protein.

Interestingly, the specific binding of LPS to human monocyte CD14 was found to be serum-dependent (6). Lipopolysaccharide binding protein (LBP) has been identified as the serum protein necessary for LPS binding to CD14 (17,21). The 70 kDa PT receptor could be a serum protein acting in a similar manner to mediate PT binding to Jurkat cells.

D. PT-biotin and the interaction of PT with cells.

PT-biotin was used according to the protocols described in Chapter V to further investigate the binding of PT to intact cells in solution. Glycoproteins and sialyllactose were used to compete for PT-biotin binding to CHO cells and Jurkat cells, which was detected using fluorescein isothiocyanate-avidin followed by immunofluorescence

microscopy. Fibrinogen at 10 and 100 times excess appeared to reduce the fluorescence resulting from PT-biotin bound to CHO cells, while sialyllactose had no detectable effect on PT-biotin binding. Fetuin (1000 times excess) eliminated the binding of PT-biotin to Jurkat cells, as shown by a complete loss of fluorescence. PT receptors on both CHO and Jurkat cells therefore appear to bind PT through a mechanism similar to serum glycoproteins. These data suggest that the inability of fetuin to affect cellular changes in the CHO cell assay result from the capacity of PT to bind irreversibly to cell surfaces (as discussed in Chapter I.C.4). The fluorescence of Jurkat cells in the presence of bound PT-biotin was unchanged or enhanced in the presence of a 1000 times excess of BSA, however, further indicating that the presence of BSA does not affect the binding of PT (see Chapter VI).

Cell-bound fluorescence is difficult to quantitate, making the interpretation of these results somewhat subjective. Presentation of the results in written form or in photographs may not be ideal. To address these concerns, it may be possible in the future to develop a more objective binding assay using CHO cells grown in 96 well microtitre plates. PT-biotin bound to these cells could be detected using avidin-alkaline phosphatase and the color development quantitated using a microtitre plate reader. Such a procedure may reveal subtle differences in PT binding to intact cells in experiments similar to those summarized above.

E. Prospects.

The ultimate goal of this research is to fully describe the mechanisms by which PT produces its biological effects in susceptible cells. Binding of the toxin to cells is only the first step in a series of events leading to modification of cellular function. Even so, much remains to be learned. For instance, it has so far been impossible to determine the affinity of PT binding to cellular receptors due to the

difficulty of obtaining ^{125}I-labeled PT at concentrations high enough to perform classical equilibrium binding assays. It is possible that a CHO cell microtitre binding assay using PT-biotin could be developed to perform binding inhibition assays similar to those described for glycoproteins in Chapter V. The information obtained may make it possible to determine the relative abilities of different cell types to bind PT, thereby offering clues about the susceptibility of these cells to the toxin *in vivo*. Though a great deal of information is now available regarding the effects of PT on cells in culture, less is known about the interactions of PT with different cells and organs in the body. Much of what we do know is measured by gross changes such as lymphocytosis (see Chapter I) that may conceal more subtle effects on cell function, such as a loss of cellular responsiveness to hormone-mediated stimulation through specific pathways (see Tables 1 and 2 in Chapter I).

After binding to a plasma membrane receptor, PT must insert into the membrane and deliver the enzymatic subunit to the cytoplasm, where it can interact with targets for ADP-ribosylation (see Chapter I). Very little is currently known about this event, though at least two lines of research are directed toward discovering the mechanism by which it occurs. The solution of the crystal structure of the toxin should provide clues about the process involved in irreversible binding and translocation, as well as specific amino acid residues involved in toxin binding. Other studies are attempting to determine whether binding of PT to glycoprotein receptors induces conformational changes required for insertion of the toxin into membrane bilayers. Together, these approaches should clear up much of the mystery pertaining to PT action.

To fully understand the various changes mediated by PT, it will be necessary to examine in more detail the cellular responses to toxin binding. This can be achieved partly by studying the effects on cells of binding of the toxin's B

oligomer. The complete characterization of PT receptors and their role in the normal function of cells will be required for full elucidation of PT's effects. This work is a first step in the development of that understanding.

Figure VII.1

Removal of photoaffinity labeled proteins from Jurkat cells with 0.5 M NaCl or 6 M guanidine hydrochloride (guanidine HCl). Jurkat cells were photoaffinity labeled using ^{125}I-ASD-PT according to the procedures in Chapter IV. PBS-washed, labeled cells were incubated for 30 min with 50 mM sodium phosphate buffer, pH 7.6, containing 0.5 M NaCl or with 6 M guanidine HCl. After centrifuging the resulting cell suspensions for 30 min at 100,000 x g, the supernatant fractions were dialyzed overnight against PBS to remove salts that might interfere with SDS-PAGE. Both the supernatant and pellet fractions were solubilized in Laemmli sample buffer and subjected to SDS-PAGE and autoradiography as described in the legend to Figure VII.1. Lanes 1 and 2 contain the pellet and supernate fractions, respectively, obtained from cells treated with 0.5 M NaCl (NaCl). Lanes 3 and 4 contain the pellet and supernate fractions, respectively, obtained from cells treated with 6 M guanidine HCl (GHCl). The arrowhead indicated the labeled 70 kDa protein and filled circles indicate autolabeled PT subunits. The positions to which the protein standards migrated are indicated on the left of each panel by a number representing their molecular weights (x 10^{-3}). The dye front and interface between the stacking and separating gels (origin) are also indicated in the figure.

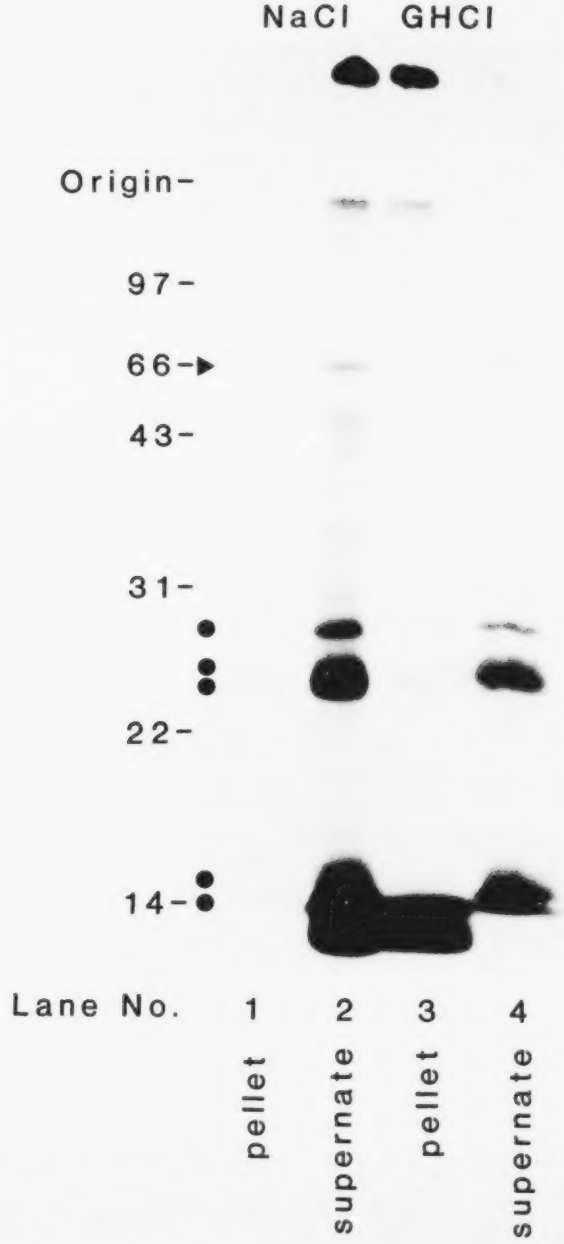

NaCl
GHCl
Origin-
97-
66-
43-
31-
22-
14-
Lane No.
1
2
3
4
pellet
supernate
pellet
supernate

Figure VII.2

Photoaffinity labeling of Jurkat cells after treatment with 0.5 M NaCl. Jurkat cells were treated for 30 min with 50 mM sodium phosphate, pH 7.4, containing 0.5 M NaCl, then washed with PBS to remove proteins released by the treatment. These treated cells were photoaffinity labeled according to the procedures in Chapter IV.B with ^{125}I-ASD-PT (lane 1), ^{125}I-APD-PT (lane 2), ^{125}I-ASD-SNA (lane 3), ^{125}I-ASD-MAL (lane 4), and ^{125}I-ASD-LPS (lane 5). The procedures used for SDS-PAGE and preparing autoradiograms were the same as those described in Figure VII.1. The 70-, 50-, and 32-kDa labeled proteins are indicated with arrowheads. Autolabeled PT subunits are indicated with filled circles, autolabeled SNA with a filled square, and autolabeled MAL subunits with stars. The positions to which the protein standards migrated are indicated on the left of each panel by a number representing their molecular weights ($\times 10^{-3}$). The dye front and interface between the stacking and separating gels (origin) are also indicated in the figure.

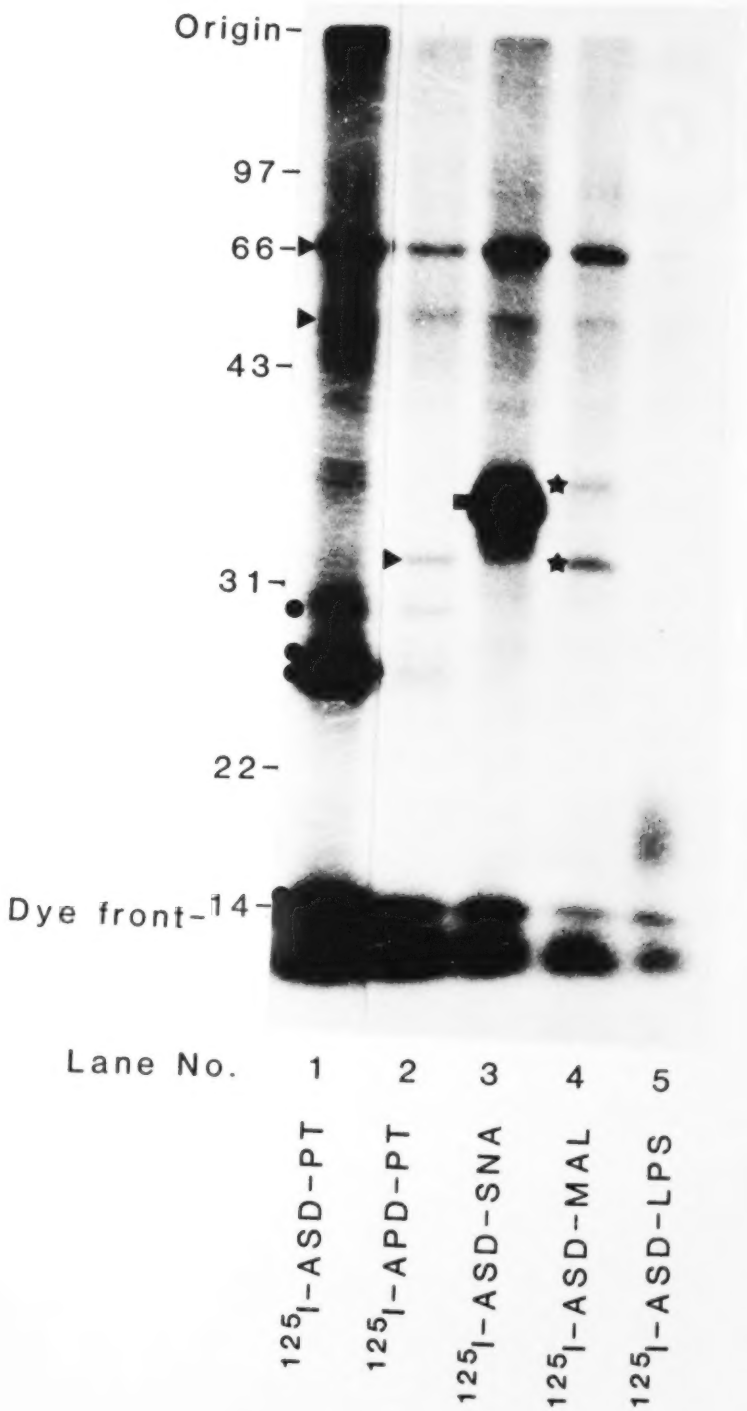
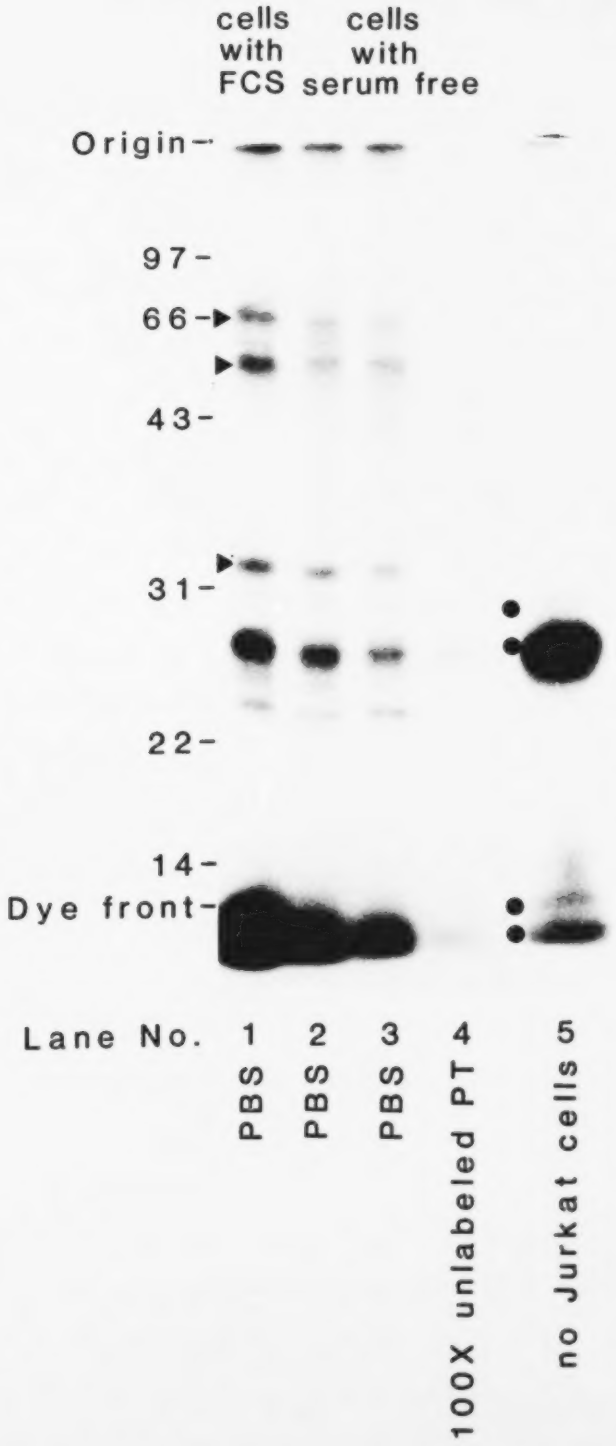

Origin-
97-
66-
43-
31-
22-
14-
Dye front-
Lane No. 1 2 3 4 5
125I-ASD-PT
125I-APD-PT
125I-ASD-SNA
125I-ASD-MAL
125I-ASD-LPS

Figure VII.3

Photoaffinity labeling of Jurkat cells grown in serum free medium with ^{125}I-APD-PT. Cells grown in medium containing 10% FCS (lane 1) or in serum free medium (lanes 2, 3, and 4) were photoaffinity labeled with ^{125}I-APD-PT using the procedures described in Chapter IV.B. Lane 3, which contained an additional 120 µl of PBS over lanes 1 and 2, was included as a control for the competition of labeling by excess unlabeled PT in lane 4 to exclude the possibility of volume effects on the labeling reaction. Lane 5 contains ^{125}I-APD-PT exposed to UV light in the absence of cells. Arrowheads indicate the 70-, 50-, and 32-kDa proteins labeled in these experiments, while filled circles indicate autolabeled PT subunits. The positions to which the protein standards migrated are indicated on the left of each panel by a number representing their molecular weights (x 10^{-3}). The dye front and interface between the stacking and separating gels (origin) are also indicated in the figure.


cells with FCS
cells with serum free
Origin
97
66
43
31
22
14
Dye front
Lane No. 1 2 3 4 5
PBS
PBS
PBS
100X unlabeled PT
no Jurkat cells

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